HIGH SALT PLANTS AND USES FOR BIOREMEDIATION

RELATED APPLICATIONS

[0001] This application is a continuation-in-part application of U.S. Application No. 10/155,535, filed May 24, 2002, which is a continuation-in-part of Application 09/271,584, filed March 18, 1999, which claims the benefit of U.S. Provisional Application No. 60/078,474, filed March 18, 1998, which are all incorporated by reference herein in their entirety. This application further claims the benefit of U.S. Provisional Applications No. 60/395,637 and No. 60/395,670, both filed July 12, 2002, which are all incorporated by reference herein in their entirety.

FIELD OF THE INVENTION

[0002] This invention is in the field of agricultural biotechnology. In particular, this invention relates to plants with elevated levels of salt stored in vacuoles and use of such plants for bioremediation with salt tolerant plants.

BACKGROUND OF THE INVENTION

[0003] Environmental stress due to salinity is one of the most serious factors limiting the productivity of agricultural crops, which are predominantly sensitive to the presence of high concentrations of salts in the soil. Large terrestrial areas of the world are affected by levels of salt inimical to plant growth. It is estimated that 35-45% of the 279 million hectares of land under irrigation is presently affected by salinity. This is exclusive of the regions classified as arid and desert lands, (which comprises 25% of the total land of our planet). Salinity has been an important factor in human history and in the life spans of agricultural systems. Salt impinging on agricultural soils has created instability and has frequently destroyed ancient and recent agrarian societies. The Sumerian culture faded as a power in the ancient world due to salt accumulation in the valleys of the Euphrates and Tigris rivers. Large areas of the Indian subcontinent have been rendered unproductive through salt accumulation and poor irrigation practices. In this century, other areas, including vast regions of Australia, Europe, southwest USA, the Canadian prairies and others have seen considerable declines in crop productivity.

[0004] Although there is engineering technology available to combat this problem, though drainage and supply of high quality water, these measures are extremely costly. In most of the cases, due to the increased need for extensive agriculture, neither improved irrigation efficiency nor the installation of drainage systems is applicable. Moreover, in the arid and semi-arid regions of the world water evaporation exceeds precipitation. These soils are inherently high in salt and require vast amounts of irrigation to become productive. Since irrigation water contains dissolved salts and minerals, an application of water is also an application of salt that compounds the salinity problem.

[0005] Increasing emphasis is being given to modify plants to fit the restrictive growing conditions imposed by salinity and even bioremediate the soil through extraction of the salt. If economically important crops could be manipulated and made salt resistant, this land could be farmed resulting in greater sales of seed and greater yield of useful crops. Conventional breeding for salt tolerance has been attempted for a long time. These breeding practices have been based mainly on the following strategies: a) the use of wide crosses between crop plants and their more salt-tolerant wild relatives, b) screening and selecting for variation within a particular phenotype, c) designing new phenotypes through recurrent selection. (Rush, et al. (1981); Norlyn (1980) and Tal (1985) The lack of success in generating tolerant varieties (given the low number of varieties released and their limited salt tolerance) would suggest that conventional breeding practices are not enough and that in order to succeed a breeding program should include the engineering of transgenic crops. (Flowers, et al. (1995) and Bonhert, et al. (1996))

[0006] Several biochemical pathways associated with stress tolerance have been characterized in different plants and a few of the genes involved in these processes have been identified and in some cases the possible role of proteins has been investigated in transgenic/overexpression experiments. Several compatible solutes have been proposed to play a role in osmoregulation under stress. Such compatible solutes, including carbohydrates, amino acids and quaternary N-compounds have been shown to increase osmoregulation under stress. (Tarcynski, et al. (1995); Kishor (1995) and Ishitani (1995)) Also, proteins that are normally expressed during seed maturation (LEAs, Late Embryogenesis Abundant proteins) have been suggested to play a role in water retention and in the protection of other proteins during stress. The overexpression of LEA in rice provided a moderate benefit to the plants during water stress.

(Xu, et al. (1996) and Wu, et al. PCT # WO/9713843) A single gene (sod2) coding for a Na+/H+ antiport has been shown to confer sodium tolerance in fission yeast. (Jia, et al. (1992) and Young, et al., PCT # WO/0106651) One of the main disadvantages of using this gene for transformation of plants is associated with the typical problems encountered in heterologous gene expression, i.e. incorrect folding of the gene product, targeting of the protein to the target membrane and regulation of the protein function.

[0007] Plants that tolerate and grow in saline environments have high intracellular salt levels. A major component of the osmotic adjustment in these cells is accomplished by ion uptake. The utilization of inorganic ions for osmotic adjustment suggests that salt-tolerant plants must be able to tolerate high levels of salts within their cells. However, enzymes extracted from these plants show high sensitivity to salt. The sensitivity of the cytosolic enzymes to salt would suggest that the maintenance of low cytosolic sodium concentration, either by compartmentation in cell organelles or by exclusion through the plasma membrane, must be necessary if the enzymes in the cell are to be protected from the inimical effects of salt.

[0008] Plant cells are structurally well suited to the compartmentation of ions. Large membrane-bound vacuoles are the site for a considerable amount of sequestration of ions and other osmotically active substances. A comparison of ion distribution in cells and tissues of various plant species indicates that a primary characteristic of salt tolerant plants is their ability to exclude sodium out of the cell and to take up sodium and to sequester it in the cell vacuoles. Transport mechanisms could actively move ions into the vacuole, removing the potentially harmful ions from the cytosol. These ions, in turn, could act as an osmoticum within the vacuole, which would then be responsible for maintaining water flow into the cell. Thus, at the cellular level both specific transport systems for sodium accumulation in the vacuole and sodium extrusion out of the cell are correlated with salt tolerance. It would be a particular advantage to use plants that accumulate salt in the vacuole in response to high salt in the soil. Such plants would accumulate the salt in the leaves and roots, which can be removed, removing a portion of the salt.

[0009] Furthermore, profits in the cattle industry are affected by the high cost of labor; thus, management procedures which reduce labor requirements are important. One management tool

frequently used is regulating feed intake with salt. Self-feeding supplements tend to allow timid, slow-eating cows to get their share and it is an easy method of providing Vitamin A, phosphorus and other feed additives. Because there are practical limits to the amount of salt cattle eat, salt can also be used to restrict the consumption of highly palatable feeds such as grain and supplement. Salt is also added to feed grain because with high grain rations, urinary calculi (phosphatic type) are a problem. This problem is controlled by feeding salt to flush out the stones. This is particularly true with milo and cottonseed meal based finishing programs. Salt supplements are added directly to the feed directly in the proportions desired. The addition and mixing requires labor which reduces profits. Thus there is a need for feed plants that already contain salt.

[0010] There is a long felt need in the art for the in situ remediation of soils damaged by accumulation of salts. The present invention enables phytoremediation and/or revegetation of contaminated environments via salt tolerant plants. The plants of the present invention may be grown in high salt soil and will accumulate salt in the leaves and roots. Such salt containing plant materials may be used as forage for cattle with the additional advantage that extra salt need not be added to the feed.

SUMMARY OF THE INVENTION

[0011] In order to meet these needs, the present invention is directed to transgenic plants that are able to grow and bioremediate soil in the presence of elevated salt concentrations. In particular, the transgenic plants remove salt from the soil and accumulate it in leaves and roots. The plants may then be harvested and fed to cattle as salt containing feed or simply removed. In a preferred embodiment of the present invention, sodium does not accumulate in the plant fruit, so the fruit is suitable for commercial sale. In particular, we show that transgenic *Brassica napus* plants overexpressing a vacuolar Na^+/H^+ antiport were able to grow, flower and produce seeds in the presence of 200 mM NaCl. *Brassica napus,* commonly known as canola or rapeseed, represents one of the most important oilseed crops that is being cultivated worldwide. The sustained growth of the transgenic plants, the seed yields and the quality of the seed oil demonstrate the potential use of these transgenic plants for bioremediation of contaminated soils.

This technology finds use in the bioremediation of soils using salt tolerant forage crops, trees and oil seed crops.

[0012] One aspect of the present invention is directed to a non-naturally occurring non-halophyte plant comprising a tissue with an elevated level of sodium substantially in the vacuole when cultivated in high salt. In one variation, the elevated salt level in the vacuole is two fold higher, three fold higher, four fold higher, five fold higher, ten fold higher, or twenty fold higher compared to the level in a comparable naturally occurring plant. In another variation, the tissue is leaf or root tissue. In yet another variation, the high salt is two fold higher, three fold higher, four fold higher, five fold higher, ten fold higher, fifteen fold higher, twenty fold higher, twenty five fold higher, or thirty fold higher than the optimal salt levels for the comparable naturally occurring plant variety. In another variation, the high salt is at or above the salt level in which the naturally occurring plant variety cannot survive. In still another variation, the plant is tomato or canola. In another variation, the cultivation in high salt conditions may be cultivation where the high salt conditions persist through the entire life cycle of the plant, the germination stage, the vegetative growth stage, the flowering stage, the seed embryogenesis stage, the stage of seed ripening, and any combination of the foregoing stages. In another variation, the plant has increase salt tolerance due to sequestering sodium in the vacuole.

[0013] Another aspect of the present invention is directed to a non-naturally occurring non-halophyte plant comprising a tissue with an enhanced level of sodium substantially in the vacuole when cultivated in high salt. In one variation, the enhanced salt level in the vacuole is two fold higher, three fold higher, four fold higher, five fold higher, ten fold higher, or twenty fold higher compared to the level in the same plant grown at low to moderate salt conditions. In another variation, the tissue is leaf or root tissue. In still another variation, the plant is tomato or canola. In another variation, the cultivation in high salt conditions may be cultivation where the high salt conditions persist through the entire life cycle of the plant, the germination stage, the vegetative growth stage, the flowering stage, the seed embryogenesis stage, the stage of seed ripening, and any combination of the foregoing stages. In another variation, the plant has increase salt tolerance due to sequestering sodium in the vacuole.

[0014] In another aspect of the present invention, the plant comprises a transgene. In one variation, the transgene comprises a first nucleic acid sequence encoding a vacuolar targeted Na+/H+ transporter or a plant derived vacuolar Na+/H+ transporter. In another variation, the transgene comprises a first nucleic acid selected from the following group: a nucleic acid molecule of the coding strand shown in SEQ ID NO:1, or a complement thereof; a nucleic acid molecule encoding the amino acid sequence shown in SEQ ID NO:2; a nucleic acid molecule that hybridizes to the sequence set forth in SEQ ID NO:1 or the complement of the sequence set forth in SEQ ID NO:1 under highly stringent conditions that include at least one wash in 0.1xSSC, 0.1% SDS, at 65° C for thirty minutes; and a nucleic acid molecule encoding a plant NHX transporter polypeptide that hybridizes to the sequence set forth in SEQ ID NO:1 or the complement of the sequence set forth in SEQ ID NO:1 under moderately stringent conditions that includes at least one wash in 0.1xSSC, 0.1% SDS, at 50° C for thirty minutes. In still another variation, the transgene further comprises a promoter sequence operably linked to the first nucleic acid sequence. In yet another variation, the promoter is a constitutive promoter or an inducible promoter. In certain variations, the promoter may be selected from the group consisting of the 35 S promoter and the CaMV promoter.

[0015] Yet another aspect of the present invention is directed to a non-naturally occurring non-halophyte plant comprising a plant with increased salt tolerance due to the ability to sequester sodium in the vacuole. Other variations exist similar to the variations discussed above.

[0016] An additional aspect of the present invention is a seed produced from any of the foregoing plants and variations thereof.

[0017] The present invention also includes methods of generating the foregoing. One variation includes transfecting a plant with a transcriptional regulatory element and identifying plants comprising seeds with normal or near normal fatty acid distribution when cultivated in high salt. In another variation, plants are transfected with a transcriptional regulatory element and identifying a plant wherein said transcriptional regulatory element has integrated operably linked to a Na+/H+ transporter. In yet another variation, the transcriptional regulatory element is a promoter, an enhancer element, a repressor element or a boundary element. In one variation, plants are transfected with a transgene comprising a Na+/H+ transporter and a plant comprising

seeds with normal or near normal fatty acid distribution when cultivated in high salt is identified. In one variation, the Na+/H+ transporter gene is selected from the group consisting of a nucleic acid molecule of the coding strand shown in SEQ ID NO:1, or a complement thereof; a nucleic acid molecule encoding the amino acid sequence shown in SEQ ID NO:2; a nucleic acid molecule that hybridizes to the sequence set forth in SEQ ID NO:1 or the complement of the sequence set forth in SEQ ID NO:1 under highly stringent conditions that include at least one wash in 0.1xSSC, 0.1% SDS, at 65° C for thirty minutes; and a nucleic acid molecule encoding a plant NHX transporter polypeptide that hybridizes to the sequence set forth in SEQ ID NO:1 or the complement of the sequence set forth in SEQ ID NO:1 under moderately stringent conditions that includes at least one wash in 0.1xSSC, 0.1% SDS, at 50° C for thirty minutes.

[0018] Another aspect of present invention is a method of lowering the salt content of soil comprising cultivating of any of the foregoing plant variations in the soil, harvesting the plant and removing the plant or the tissue with an elevated level of sodium or an enhanced level of sodium. In one variation, the electrical conductivity of the soil is at least 15 dS/m, at least 20 dS/m, at least 25 dS/m, at least 30 dS/m, at least 40 dS/m, or at least 50 dS/m. In another variation, the harvesting step is omitted from the method.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] **Figure 1** shows salt tolerance of wild-type tomato plants and transgenic plants overexpressing AtNHX1grown in the presence of 200 mM NaCl. (A) wild-type plants grown in the presence of 5 mM NaCl. (B) transgenic plants overexpressing AtNHX1, grown in the presence of 5 mM NaCl. (C) Western blots from leaf membrane proteins (5 μg) tested with antibodies raised against AtNHX1. Upper panel: Lanes 1 and 4, tonoplast-enriched fraction; lanes 2 and 5, Golgi/ER-enriched fractions; 3 and 6, plasma membrane fraction. Lanes 1,2,3 correspond to membranes from wild-type plants while lanes 4,5,6 correspond to membranes from transgenic plants. Relative molecular masses are indicated on the left; lower panel: Enrichment of the fractions with tonoplast membranes was assessed with antibodies raised against the vacuolar H+-PPiase. (D) wild-type plants grown in the presence of 200 mM NaCl. (E)) transgenic plants overexpressing AtNHX1, grown in the presence of 200 mM NaCl. Plants shown after 11 weeks of growth.

[0020] Bar = 25 cm.

[0021] **Figure 2** shows Na+/H+ exchange activity in leaf tonoplast vesicles Membrane fractions were purified from leaves using the method described5 with the modifications described4. At the indicated times, the vacuolar H+-PPiase was activated by the addition of Mg2+. When a steady-state pH gradient (acidic inside) was formed, the PPi-dependent H+ transport activity was stopped by the addition of AMDP and the rates of cation/H+ exchange were determined in vesicles isolated from wild-type plants (WT) and transgenic plants overexpressing AtNHX1 (X1OE). (A) Na+-dependent H+ exchange, (B) K+-dependent H+ exchange. The addition of monensin (mon), an artificial Na+/H+ antiport, or nigericin (nig), an artificial K+/H+ antiport, abolished the pH gradient and the fluorescence was fully recovered. The figure shows a typical recording.

[0022] **Figure 3** shows ion, sugar, and proline contents of wild-type and transgenic plants grown at different salt concentrations. Wild-type (hatched line bars) and transgenic plants (cross-hatched line bars) grown in the presence of 5 mM NaCl. Two independent transgenic lines (black and white bars) grown in the presence of 200 mM NaCl. (A) Na+ contents; (B) K+ contents; (C) Cl—contents; (D) soluble sugar contents; (E) proline contents. For each determination, leaves, roots and fruits from ten plants were collected from each hydroponic tank and pooled. Values are the Mean Δ S.D. from material collected from three hydroponic tanks (n = 3).

[0023] **Figure 4** shows fruits from wild-type and transgenic plants. (A) tomato fruits from wild-type plants; (B) tomato fruits from transgenic plants. (C) Western blots from fruit tonoplast proteins (5 μg) tested with antibodies raised against AtNHX1. Wild-type plants grown in the presence of 5 mM NaCl (lane 1). Two independent transgenic lines grown in the presence of 200 mM NaCl (lanes 2 and 3).

[0024] **Figure 5** shows salt tolerance of wild-type plants and transgenic *Brassica* plants overexpressing AtNHX1grown in the presence of 200 mM NaCl. Wild-type (wt) and homozygous plants showing high ($X1OE_1$), medium ($X1OE_2$) and low ($X1OE_3$) levels of expression were grown in the presence of 200 mM NaCl. Plants shown after 10 weeks of growth. *Inset:* Western blots of leaf tonoplast-enriched membrane fractions isolated from wild-

type and transgenic plants with low, medium and high levels of expression of *AtNHX1*. Blots were probed with antibodies raised against the C-terminus of AtNHX1. Equal amounts of protein (20 μg) were loaded in each lane. Relative molecular masses are indicated on the left.

[0025] **Figure 6** shows Na^+ and K^+ contents of leaves and roots from wild-type plants grown at 10 mM NaCl (black bars) and transgenic plants (X1OE1) grown at 10 mM NaCl (white bars) and 200 mM NaCl (hatched line bars). **(A)** Na^+ content; **(B)** K^+ content. Leaves and roots were collected from fifteen plants from each treatment, the material pooled in three groups and ion contents measured as described in Materials and Methods. Values are the Mean ±S.D (n = 3).

[0026] **Figure 7** shows proline, soluble sugars, protein and total nitrogen contents of leaves and roots from wild-type plants grown at 10 mM NaCl (black bars); and transgenic plants ($X1OE_1$) grown at 10 mM NaCl (white bars) and 200 mM NaCl (hatched line bars). **(A)** Proline content; **(B)** soluble sugar content; **(C)** total protein content; **(D)** total nitrogen content. Leaves and roots were collected from fifteen plants from each treatment, the material pooled in three groups and contents measured as described in Materials and Methods. Values are the Mean ±S.D (n = 3).

[0027] **Figure 8** shows fatty acid composition of the minor chloroplastic lipids from wild-type plants grown at 10 mM NaCl (black bars); and transgenic plants grown ($X1OE_1$) at 10 mM NaCl (white bars) and 200 mM NaCl (hatched line bars). **(A)** Sulfoquinovosyldiacylglycerol; **(B)** Phosphatidylglycerol. Leaves were collected as leaf discs from 15 plants from each treatment, the material pooled in to 3 groups of 2 g each and contents purified and measured as described in Material and Methods. Values are the Mean ±S.D (n = 5).

[0028] **Figure 9** shows fatty acid composition of seeds from wild-type plants grown in 10 mM NaCl (black bars) and transgenic plants ($X1OE_1$) grown in the presence of 200 mM NaCl (hatched line bars). Seeds were collected from individual plants and batches of 3 seeds per plant were used for each measurement. Values are the Mean ±S.D (n =5).

BRIEF DESCRIPTION OF THE TABLES

[0029] **Table I** shows a comparison of the yield of a non-naturally occurring salt tolerant oil crop in the presence of 10 mM NaCl and 200 mM NaCl and the yield of the naturally occurring oil crop of the same variety grown in the presence of 10 mM NaCl.

[0030] **Table II** shows the a comparison of the lipid content of leaves and roots of a non-naturally occurring salt tolerant oil crop grown in the presence of 10 mM and 200 mM NaCl and a naturally occurring oil crop of the same variety grown in the presence of 10 mM NaCl.

[0031] **Table III** shows a representative list of NXH related gene products.

[0032] **Table IV** shows the plant and fruit yield of a non-naturally occurring non-halophyte tomato plant grown in the presence of 5 mM and 200 mM NaCl and a naturally occurring non-halophyte tomato plant of the same variety grown in the presence of 5 mM NaCl.

[0033] **Table V** shows the salinity levels that lead to a 25% relative decrease in yield and a 50% relative decrease in yield for various crop plants, including soybean, an oil crop plant.

DETAILED DESCRIPTION OF THE INVENTION

[0034] The present invention provides a non-naturally occurring plant that is characterized by increased salt tolerance due to sequestering salt in the vacuole. A preferred method of generating such plants is by ectopic expression of a nucleic acid molecule encoding an NHX related gene product that finds use in bioremediation. The NHX related gene product can have, for example, substantially the amino acid sequence of an NHX ortholog such as those described in Table III.

[0035] In one embodiment, the invention provides a transgenic plant characterized by increased salt tolerance due to ectopic expression of an exogenous nucleic acid molecule encoding an NHX-related gene product. The nucleic acid molecule encoding the NHX-related gene product can be operatively linked to an exogenous regulatory element such as a constitutive regulatory element or crop-selective regulatory element.

[0036] The present invention is directed to the surprising discovery that the NHX increases salt tolerance in plants. As disclosed herein, transgenic *Brassica* plants overexpressing AtNHX1 were able to grow, flower and produce seeds in the presence of 200 mM NaCl. Furthermore, as disclosed in Example 2, *Lycopersicon esculentum* plants overexpressing AtNHX1 were also able to grow, flower and produce fruit in the presence of 200 mM NaCl. The fruit produced had near normal levels of sodium and was thus suitable for commercial sale.

[0037] As further disclosed herein, overexpression of AtNHX1 in *Brassica* plants results in increased salt tolerance as compared to the salt tolerance of naturally occurring *Brassica* plants. As set forth in the Examples, constitutive expression of NHX1 under control of a 35 S promoter resulted in plants having increased salt tolerance as compared to the salt tolerance of naturally occurring plants. In view of the presence and expression of the NHX ortholog, as detailed in Table III, the skilled artisan will recognize that an NHX-related gene product, such as an ortholog of NHX, can be used in the methods of the present invention, for example, to produce transgenic plants having the characteristics disclosed herein. Thus, the invention provides a non-naturally occurring plant such as a transgenic *Brassica* plant, characterized by increased salt tolerance due to ectopic expression of a nucleic acid molecule encoding an NHX related gene product.

[0038] As used herein, the term "non-naturally occurring," when used in reference to a plant, means a plant that has been genetically modified by human intervention. A transgenic plant of the invention, for example, is a non-naturally occurring plant that contains an exogenous nucleic acid molecule, such as a nucleic acid molecule encoding an NHX-related gene product and, therefore, has been genetically modified by human intervention. In addition, a plant that contains, for example, a mutation in an endogenous NHX-related gene product regulatory element or coding sequence as a result of calculated exposure to a mutagenic agent, such as a chemical mutagen, or an "insertional mutagen," such as a transposon, also is considered a non-naturally occurring plant, since it has been genetically modified by human intervention. Furthermore, a plant generated by cross breeding different strains and varieties are also considered a "non-naturally occurring plant," because the selection and breeding is performed by human intervention. In contrast, a plant containing only spontaneous or naturally occurring mutations is not a "non-naturally occurring plant" as defined herein and, therefore, is not

encompassed within the invention. Wild type plants are examples of naturally occurring plants. One skilled in the art understands that, while a non-naturally occurring plant typically has a nucleotide sequence that is altered as compared to a similar naturally occurring plant, a non-naturally occurring plant also can be genetically modified by human intervention without altering its nucleotide sequence, for example, by modifying its methylation pattern.

[0039] The term "ectopically," as used herein in reference to expression of a nucleic acid molecule, refers to an expression pattern in a non-naturally occurring plant that is distinct from the expression pattern in a comparable naturally occurring plant. Thus, one skilled in the art understands that ectopic expression of a nucleic acid molecule encoding an NHX-related gene product can refer to expression in a cell type other than a cell type in which the nucleic acid molecule normally is expressed, or at a time other than a time at which the nucleic acid molecule normally is expressed, or at a level other than the level at which the nucleic acid molecule normally is expressed. For example, under control of a constitutive promoter such as the cauliflower mosaic virus 35S promoter, NHX is expressed is expressed at higher than normal levels in plants and, thus, is ectopically expressed.

[0040] The term "increased salt tolerance," as used herein in reference to a non-naturally occurring plant variety of the invention, means a significantly increased salt tolerance as compared to the salt tolerance of a corresponding plant variety lacking a genetic modification introduced by human intervention such as an ectopically expressed nucleic acid molecule encoding an NHX-related gene product. As disclosed herein in the Examples, transgenic *Brassica napus* plants and transgenic tomato plants ectopically expressing NHX-1 (both examples of non-naturally occurring plants) have an increased salt tolerance as compared to naturally occurring *Brassica* plants and naturally occurring tomato plants, respectively.

[0041] It is recognized that there can be natural variation in the salt tolerance of a particular plant species or variety. However, the salt tolerance of a plant using a method of the invention readily can be identified by sampling a population of the plant and determining that the normal distribution of salt tolerance is higher, on average, than the normal distribution of a plant lacking an ectopically expressed nucleic acid molecule encoding an NHX-related gene product. Thus, production of non-naturally occurring plant varieties of the invention provides a means to skew

the normal distribution of salt tolerance of a plant, such that the salt tolerance is, on average, at least about 5% greater, 10% greater, 20% greater, 30% greater, 50% greater, 75% greater, 100% greater, 200% greater, 300% greater, 400% greater or 500% greater than in the corresponding naturally plant variety.

[0042] The term "elevated level of sodium" in vacuoles within a plant tissue, as used herein in reference to a non-naturally occurring plant variety of the invention, means an increased concentration of sodium in the vacuole and not the cytoplasm as compared to the salt concentration of a corresponding plant variety under the same salinity and lacking a genetic modification introduced by human intervention such as an ectopically expressed nucleic acid molecule encoding an NHX-related gene product. As disclosed herein in the Examples, transgenic *Brassica napus* plants and transgenic tomato plants ectopically expressing NHX-1 sequester sodium in the vacuoles of their root and leaf tissue and thus have elevated levels of sodium as compared to naturally occurring *Brassica* plants and naturally occurring tomato plants, respectively.

[0043] It is recognized that there can be natural variation in the salt levels of a particular plant species or variety in the cytoplasm and vacuole. However, the relative salt concentrations within a plant using a method of the invention can be identified by sampling a population of the plant and determining that the ratio of salt in the vacuole as compared to the cytoplasm is higher, on average, than the ratio in a naturally occurring plant of the same variety. See for example Carden *et al.* (2001) and Carden *et al.* (2003). Thus, production of non-naturally occurring plant varieties of the invention provides a means to skew the normal distribution of salt between the vacuole and the cytoplasm when grown under increased salt conditions, such that the ratio of sodium in the vacuole to sodium in the cytoplasm is, on average, at least about 5% greater, 10% greater, 20% greater, 30% greater, 50% greater, 75% greater, 100% greater, 200% greater, 300% greater, 400% greater or 500% greater than in the corresponding naturally occurring plant variety.

[0044] The term "enhanced sodium levels" in vacuoles within a plant tissue, as used herein in reference to a non-naturally occurring plant variety of the invention, means an increased concentration of sodium in the vacuole and not the cytoplasm as compared to the salt

concentration of the same plant variety grown in low to moderate salinity. As disclosed herein in the Examples, transgenic *Brassica napus* plants and transgenic tomato plants ectopically expressing NHX-1 sequester sodium in the vacuoles of their root and leaf tissue and thus have enhanced levels of sodium when grown at 200 mM NaCl as compared to the same transgenic plants grown at 10 mM NaCl.

[0045] It is recognized that there can be natural variation in the salt levels of a particular plant species or variety in the cytoplasm and vacuole. However, the relative salt concentrations within a plant using a method of the invention can be identified by sampling a population of the plant and determining that the ratio of salt in the vacuole as compared to the cytoplasm is higher, on average, than the ratio in a naturally occurring plant of the same variety. See for example Carden *et al.* (2001) and Carden *et al.* (2003). Thus, production of non-naturally occurring plant varieties of the invention provides a means to skew the normal distribution of salt between the vacuole and the cytoplasm when grown under increased salt conditions, such that the ratio of sodium in the vacuole to sodium in the cytoplasm is, on average, at least about 5% greater, 10% greater, 20% greater, 30% greater, 50% greater, 75% greater, 100% greater, 200% greater, 300% greater, 400% greater or 500% greater than in the same plant grown under low to moderate salt conditions.

[0046] The term "non-halophyte," as used herein means a plant that is not naturally morphologically and/or physiologically adapted to grow in salt rich soils or salt laden air. A non-halophyte is a plant variety that has a relative yield decrease of 50 % or more at 200 mM NaCl (the equivalent of about 20 dS/m) when compared to the plant variety grown at optimal salinity levels which are below 200 mM NaCl. For the avoidance of doubt, a non-naturally occurring non-halophyte may have a relative yield decrease of less than 50% in the presence of 200 mM NaCl due to the human introduced genetic modification of the plant. The essential part of the definition is that the plant does not *naturally* tolerate salinity well. The invention is suitable for even more salt sensitive naturally occurring plant varieties which have a relative yield decrease of 50% or more at 180 mM NaCl, 160 mM NaCl, 140 mM NaCl, 120 mM NaCl, 100 mM NaCl or 80 mM NaCl. Table IV lists the relative yield decrease for various non-halophyte crop plants.

[0047] The term "saline-intolerant plants" as used herein means a plant variety that cannot complete its life cycle in growth media containing a salinity level above 200 mM NaCl. The invention is suitable for even more highly saline-intolerant plant varieties that cannot complete their life cycle in growth media containing a salinity level above 180 mM NaCl, 160 mM NaCl, 140 mM NaCl, 120 mM NaCl, 100 mM NaCl and even 7 mM NaCl.

Methods of Making the Plants

[0048] The following methods are illustrative of some of the methods that may be used to make the plants of the present invention. With the Examples herein, one of skill in the art will now recognize that many methods may be used to generate the non-naturally occurring plants of the present invention based upon dealing with salt accumulation in the cytosol by sequestering the salt in the plant's vacuole. A preferred method is generating a plant ectopically expressing an NHX-related gene product targeted to the plant's vacuole. From this disclosure, it will now be apparent that any sodium transporter may be used by the addition of targeting sequences that result in localization to the vacuolar membrane.

[0049] As used herein, the term "NHX-related gene product" means a gene product that has the same or similar function as At NHX such that, when ectopically expressed in a plant, normal salt tolerance is altered such that plants with increased salt tolerance are produced. *Arabidopsis* NHX-1 is an example of an NHX-related gene product as defined herein.

[0050] An NHX-related gene product generally is characterized, in part, as containing a putative cation binding domain and an amiloride binding domain. An NHX-related gene product also generally is characterized by having an amino acid sequence that has at least about 40% amino acid identity with the amino acid sequence of *Arabidopsis* NHX-1. An NHX-related gene product can have, for example, an amino acid sequence with greater than about 45% amino acid sequence identity with *Arabidopsis* NHX-1, preferably greater than about 50% amino acid identity with *Arabidopsis* NHX-1, more preferably greater than about 55% amino acid sequence identity with Arabidopsis NHX-1, preferably greater than about 60% amino acid identity with Arabidopsis NHX-NX-1, preferably greater than about 65% amino acid sequence identity with Arabidopsis NHX-1, preferably greater than about 75% amino acid identity with Arabidopsis NHX-1, more preferably greater than about 85% amino acid identity with Arabidopsis NHX-1,

and can be a sequence having greater than about 90%, 95% or 97% amino acid identity with *Arabidopsis* NHX-1.

[0051] Preferably, an NHX-related gene product is orthologous to the plant species in which it is ectopically expressed. A nucleic acid molecule encoding *Brassica* NHX, for example, can be ectopically expressed in a *Brassica* plant to produce a non-naturally occurring *Brassica* variety characterized by an increased salt tolerance. Similarly, a nucleic acid molecule encoding oil plant NHX, for example, can be ectopically expressed in a plant to produce a non-naturally occurring plant characterized by producing salt tolerant plants.

[0052] A nucleic acid molecule encoding an NHX-related gene product also can be ectopically expressed in a heterologous plant to produce a non-naturally occurring plant characterized by an increased salt tolerance. NHX proteins have been cloned from a number of plant species (including monocots such as Arabidopsis, tomato, sugar beets, petunia, as well as monocots such as rice (see e.g. U.S. Application No. 09/888,035, filed June 22, 2001, herein incorporated by reference), etc.) indicating that they are widely conserved throughout the plant species. NHX-related gene products such as NHX orthologs also can be conserved and can function across species boundaries to result in an increased salt tolerance. Thus, ectopic expression of a nucleic acid molecule encoding NHX in a heterologous plant can alter the salt tolerance of the plant. Furthermore, a nucleic acid molecule encoding a vacuole targeted NHX-related gene product, for example, can be ectopically expressed in more distantly related heterologous plants, including plants, and, upon ectopic expression, can alter salt tolerance.

[0053] As used herein, the term "NHX-related gene product" encompasses an active segment of an NHX-related gene product, which is a polypeptide portion of an NHX-related gene product that, when ectopically expressed, increases salt tolerance. An active segment can be, for example, an amino terminal, internal or carboxy terminal fragment of NHX-1 that, when ectopically expressed in a plant, results in an increased salt tolerance. The skilled artisan will recognize that a nucleic acid molecule encoding an active segment of an NHX-related gene product can be used to generate a plant of the invention characterized by an increased salt tolerance and in the related methods and kits of the invention described further below.

[0054] An active segment of an NHX-related gene product can be identified using the methods described in The Example or using other routine methodology. Briefly, a plant such as *Brassica napus* can be transformed with a nucleic acid molecule under control of a constitutive regulatory element such as a tandem CaMV 35S promoter. Biochemical analysis of the plant and plant growth observations reveals whether a plant ectopically expressing a particular polypeptide portion has an increased salt tolerance. For analysis of a large number of polypeptide portions of an NHX-related gene product, nucleic acid molecules encoding the polypeptide portions can be assayed in pools, and active pools subsequently subdivided to identify the active nucleic acid molecule.

[0055] In one embodiment, the invention provides a non-naturally occurring plant that is characterized by an increased salt tolerance due to ectopic expression of a nucleic acid molecule encoding an NHX-related gene product having substantially the amino acid sequence of an NHX ortholog. As used herein, the term "NHX ortholog" means an ortholog of Arabidopsis NHX-1 and refers to an NHX-related gene product that, in a particular plant variety, has the highest percentage homology at the amino acid level to Arabidopsis NHX-1. An NHX-1 ortholog can be, for example the NHX-1 orthologs described in Table III. Novel NHX ortholog cDNAs can be isolated from additional plant species using a nucleotide sequence as a probe and methods well known in the art of molecular biology (Glick and Thompson (eds.), Methods in Plant Molecular Biology and Biotechnology, Boca Raton, Fla.: CRC Press (1993); Sambrook et al. (eds.), Molecular Cloning: A Laboratory Manual (Second Edition), Plainview, N.Y.: Cold Spring Harbor Laboratory Press (1989), each of which is incorporated herein by reference).

[0056] As used herein, the term "substantially the amino acid sequence," when used in reference to an NHX ortholog, is intended to mean a polypeptide or polypeptide segment having an identical amino acid sequence, or a polypeptide or polypeptide segment having a similar, non-identical sequence that is considered by those skilled in the art to be a functionally equivalent amino acid sequence. For example, an NHX-related gene product having substantially the amino acid sequence of Arabidopsis NHX-1 can have an amino acid sequence identical to the sequence of Arabidopsis NHX-1, or a similar, non-identical sequence that is functionally equivalent. In particular, a gene product that has "substantially the amino acid sequence" of an NHX ortholog can have one or more modifications such as amino acid additions, deletions or substitutions,

including conservative or non-conservation substitutions, relative to the NHX-1 amino acid sequence, for example, provided that the modified polypeptide retains substantially the ability to increase salt tolerance when the nucleic acid molecule is ectopically expressed in the plant. Comparison of sequences for substantial similarity can be performed between two sequences of any length and usually is performed with sequences between about 6 and 1200 residues, preferably between about 10 and 100 residues and more preferably between about 25 and 35 residues. Such comparisons for substantial similarity are performed using methodology routine in the art.

[0057] The preferred percentage of sequence similarity for sequences of NHX orthologs includes nucleotide sequences having at least about: 48% similarity to SEQ ID NO:1. The similarity may also be at least about: 60% similarity, 75% similarity, 80% similarity, 90% similarity, 95% similarity, 97% similarity, 98% similarity, 99% similarity, or more preferably at least about 99.5% similarity, wherein the polypeptide has Na+/H+ transporter activity. The invention also includes salt tolerant plants made by transgenic expression of nucleic acid molecules encoding polypeptides, with the polypeptides having at least about: at least about: 48% similarity to SEQ ID NO:2. The similarity may also be at least about: 60% similarity, 75% similarity, 80% similarity, 90% similarity, 95% similarity, 97% similarity, 98% similarity, 99% similarity, or more preferably at least about 99.5% similarity, wherein the polypeptide Na+/H+ has transporter activity, to SEQ ID NO:2 (or a partial sequence thereof) considering conservative amino acid changes, wherein the polypeptide has Na+/H+ transporter activity. Sequence similarity is preferably calculated as the number of similar amino acids in a pairwise alignment expressed as a percentage of the shorter of the two sequences in the alignment. The pairwise alignment is preferably constructed using the Clustal W program, using the following parameter settings: fixed gap penalty=10, floating gap penalty=10, protein weight matrix=BLOSUM62. Similar amino acids in a pairwise alignment are those pairs of amino acids which have positive alignment scores defined in the preferred protein weight matrix (BLOSUM62). The protein weight matrix BLOSUM62 is considered appropriate for the comparisons described here by those skilled in the art of bioinformatics. (The reference for the clustal w program (algorithm) is Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22:4673-4680; and the reference

for BLOSUM62 scoring matrix is Henikoff, S. and Henikoff, J.G. (1993) Performance evaluation of amino acid substitution matrices. Proteins, 7:49-61.)

[0058] It is understood that minor modifications of primary amino acid sequence can result in an NHX-related gene product that has substantially equivalent or enhanced function as compared to the NHX ortholog from which it was derived. Further, various molecules can be attached to an NHX ortholog or active segment thereof, for example, other polypeptides, antigenic or other peptide tags, carbohydrates, lipids, or chemical moieties. Such modifications are included within the term NHX ortholog as defined herein.

[0059] One or more point mutations can be introduced into a nucleic acid molecule encoding an NHX ortholog to yield a modified nucleic acid molecule using, for example, site-directed mutagenesis (see Wu (Ed.), Meth. In Enzymol. Vol. 217, San Diego: Academic Press (1993); Higuchi, "Recombinant PCR" in Innis et al. (Ed.), PCR Protocols, San Diego: Academic Press, Inc. (1990), each of which is incorporated herein by reference). Such mutagenesis can be used to introduce a specific, desired amino acid insertion, deletion or substitution; alternatively, a nucleic acid sequence can be synthesized having random nucleotides at one or more predetermined positions to generate random amino acid substitutions. Scanning mutagenesis also can be useful in generating a modified nucleic acid molecule encoding substantially the amino acid sequence of an NHX ortholog.

[0060] Modified nucleic acid molecules can be routinely assayed for the ability to alter normal plant development such that salt tolerance is increased. For example, a nucleic acid molecule encoding substantially the amino acid sequence of an NHX ortholog can be ectopically expressed, for example, using a constitutive regulatory element such as the CaMV 35S promoter or using a tissue-specific regulatory element such as a seed-selective regulatory element as described further below. If such ectopic expression results in a seed plant in which seeds of increased size are produced, the modified polypeptide or segment is an "NHX ortholog" as defined herein.

[0061] Other functional equivalent forms of the NHX-related gene product encoding nucleic acids can be identified using conventional DNA-DNA or DNA-RNA hybridization techniques.

These nucleic acid molecules and the AtNHX sequences can be modified without significantly affecting their activity.

[0062] The plants of the present invention may therefore also be made by generating transgenic plants containing nucleic acid molecules that hybridize to one SEQ ID NO:1 or their complementary sequences, and that encode expression for peptides or polypeptides exhibiting substantially equivalent activity as that of an AtNHX polypeptide produced by SEQ ID NO:1 or their variants. Such nucleic acid molecules preferably hybridize to the sequences under low, moderate (intermediate), or high stringency conditions. (see Sambrook et al. (Most recent edition) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

[0063] As used herein, the phrase "low stringency hybridization conditions" refers the following conditions and equivalents thereto: hybridization at 5xSSC, 2% SDS, and 100 μg/ml single stranded DNA at 40° C for 8 hours, followed by at least one wash in 2xSSC, 0.2% SDS, at 40° C for thirty minutes.

[0064] As used herein, the phrase "moderate stringency hybridization conditions" refers the following conditions and equivalents thereto: hybridization at 5xSSC, 2% SDS, and 100 μg/ml single stranded DNA at 50° C for 8 hours, followed by at least one wash in 0.1xSSC, 0.1% SDS, at 50° C for thirty minutes.

[0065] As used herein, the phrase "high stringency hybridization conditions" refers the following conditions and equivalents thereto: hybridization at 5xSSC, 2% SDS, and 100 μg/ml single stranded DNA at 65° C for 8 hours, followed by at least one wash in 0.1xSSC, 0.1% SDS, at 65° C for thirty minutes.

[0066] The invention also provides a transgenic plant that is characterized by increased salt tolerance resulting from ectopic expression of an exogenous nucleic acid molecule encoding an NHX-related gene product targeted to plant vacuoles. In a transgenic plant of the invention, the ectopically expressed exogenous nucleic acid molecule encoding an NHX-related gene product can be operatively linked to an exogenous regulatory element. In one embodiment, the invention provides a transgenic plant characterized by increased salt tolerance having an ectopically

expressed exogenous nucleic acid molecule encoding an NHX-related gene product that is operatively linked to a constitutive regulatory element. The invention provides, for example, a transgenic plant that is characterized by an increased salt tolerance due to ectopic expression of an exogenous nucleic acid molecule encoding substantially the amino acid sequence of an NHX ortholog operatively linked to a cauliflower mosaic virus 35S promoter.

[0067] In another embodiment, an exogenous constitutive or inducible regulatory element may be introduced to the plant such that the exogenous regulatory element is operably linked to an endogenous gene and alters the expression pattern of the gene in a manner that provides salt tolerance due to sequestering salt in the vacuole. One example of this would be to transfect a plant with the cauliflower mosaic virus 35S promoter such that the promoter integrates in a way that it is operably linked to one of the plant's endogenous NHX-related genes.

[0068] In yet another embodiment, an exogenous NHX-related gene may be introduced to the plant such that the exogenous NHX-related gene is operably linked to an endogenous regulatory element which directs the expression of the gene in a manner that provides salt tolerance due to sequestering salt in the vacuole. One example of this would be to transfect a plant with the atNHX1 gene such that the gene integrates in a way that it is operably linked to one of the plant's endogenous strong promoters.

[0069] As used herein, the term "transgenic" refers to a plant that contains an exogenous nucleic acid molecule, which can be derived from the same plant species or from a heterologous plant species.

[0070] The term "exogenous," as used herein in reference to a nucleic acid molecule and a transgenic plant, means a nucleic acid molecule originating from outside the plant. An exogenous nucleic acid molecule can have a naturally occurring or non-naturally occurring nucleotide sequence. One skilled in the art understands that an exogenous nucleic acid molecule can be a heterologous nucleic acid molecule derived from a different plant species than the plant into which the nucleic acid molecule is introduced or can be a nucleic acid molecule derived from the same plant species as the plant into which it is introduced.

[0071] The term "operatively linked," as used in reference to a regulatory element and a nucleic acid molecule, such as a nucleic acid molecule encoding an NHX-related gene product, means that the regulatory element confers regulated expression upon the operatively linked nucleic acid molecule. Thus, the term "operatively linked," as used in reference to an exogenous regulatory element such as a constitutive regulatory element and a nucleic acid molecule encoding an NHX-related gene product, means that the constitutive regulatory element is linked to the nucleic acid molecule encoding an NHX-related gene product such that the expression pattern of the constitutive regulatory element is conferred upon the nucleic acid molecule encoding the NHX-related gene product. It is recognized that a regulatory element and a nucleic acid molecule that are operatively linked have, at a minimum, all elements essential for transcription, including, for example, a TATA box.

[0072] As used herein, the term "constitutive regulatory element" means a regulatory element that confers a level of expression upon an operatively linked nucleic molecule that is relatively independent of the cell or tissue type in which the constitutive regulatory element is expressed. A constitutive regulatory element that is expressed in a plant generally is widely expressed in a large number of cell and tissue types.

[0073] A variety of constitutive regulatory elements useful for ectopic expression in a transgenic plant of the invention are well known in the art. The cauliflower mosaic virus 35S (CaMV 35S) promoter, for example, is a well-characterized constitutive regulatory element that produces a high level of expression in all plant tissues (Odell et al., Nature 313:810-812 (1985)). The CaMV 35S promoter can be particularly useful due to its activity in numerous diverse plant species (Benfey and Chua, Science 250:959-966 (1990); Futterer et al., Physiol. Plant 79:154 (1990); Odell et al., supra, 1985). A tandem 35S promoter, in which the intrinsic promoter element has been duplicated, confers higher expression levels in comparison to the unmodified 35S promoter (Kay et al., Science 236:1299 (1987)). Other constitutive regulatory elements useful for ectopically expressing a nucleic acid molecule encoding an NHX-related gene product in a transgenic plant of the invention include, for example, the cauliflower mosaic virus 19S promoter; the Figwort mosaic virus promoter; and the nopaline synthase (nos) gene promoter (Singer et al., Plant Mol. Biol. 14:433 (1990); An, Plant Physiol. 81:86 (1986)).

[0074] Additional constitutive regulatory elements including those for efficient ectopic expression in monocots also are known in the art, for example, the pEmu promoter and promoters based on the rice Actin-1 5' region (Last et al., Theor. Appl. Genet. 81:581 (1991); Mcelroy et al., Mol. Gen. Genet. 231:150 (1991); Mcelroy et al., Plant Cell 2:163 (1990)). Chimeric regulatory elements, which combine elements from different genes, also can be useful for ectopically expressing a nucleic acid molecule encoding an NHX-related gene product (Comai et al., Plant Mol. Biol. 15:373 (1990)). One skilled in the art understands that a particular constitutive regulatory element is chosen based, in part, on the plant species in which a nucleic acid molecule encoding an NHX-related gene product is to be ectopically expressed and on the desired level of expression.

[0075] An exogenous regulatory element useful in a transgenic plant of the invention also can be an inducible regulatory element, which is a regulatory element that confers conditional expression upon an operatively linked nucleic acid molecule, where expression of the operatively linked nucleic acid molecule is increased in the presence of a particular inducing agent or stimulus as compared to expression of the nucleic acid molecule in the absence of the inducing agent or stimulus. Particularly useful inducible regulatory elements include copper-inducible regulatory elements (Mett et al., Proc. Natl. Acad. Sci. USA 90:4567-4571 (1993); Furst et al., Cell 55:705-717 (1988)); tetracycline and chlor-tetracycline-inducible regulatory elements (Gatz et al., Plant J. 2:397-404 (1992); Roder et al., Mol. Gen. Genet. 243:32-38 (1994); Gatz, Meth. Cell Biol. 50:411-424 (1995)); ecdysone inducible regulatory elements (Christopherson et al., Proc. Natl. Acad. Sci. USA 89:6314-6318 (1992); Kreutzweiser et al., Ecotoxicol. Environ. Safety 28:14-24 (1994)); heat shock inducible regulatory elements (Takahashi et al., Plant Physiol. 99:383-390 (1992); Yabe et al., Plant Cell Physiol. 35:1207-1219 (1994); Ueda et al., Mol. Gen. Genet. 250:533-539 (1996)); and lac operon elements, which are used in combination with a constitutively expressed lac repressor to confer, for example, IPTG-inducible expression (Wilde et al., EMBO J. 11:1251-1259 (1992)).

[0076] An inducible regulatory element useful in the transgenic plants of the invention also can be, for example, a nitrate-inducible promoter derived from the spinach nitrite reductase gene (Back et al., Plant Mol. Biol. 17:9 (1991)) or a light-inducible promoter, such as that associated with the small subunit of RuBP carboxylase or the LHCP gene families (Feinbaum et al., Mol.

Gen. Genet. 226:449 (1991); Lam and Chua, Science 248:471 (1990)). Additional inducible regulatory elements include salicylic acid inducible regulatory elements (Uknes et al., Plant Cell 5:159-169 (1993); Bi et al., Plant J. 8:235-245 (1995)); plant hormone-inducible regulatory elements (Yamaguchi-Shinozaki et al., Plant Mol. Biol. 15:905 (1990); Kares et al., Plant Mol. Biol. 15:225 (1990)); and human hormone-inducible regulatory elements such as the human glucocorticoid response element (Schena et al., Proc. Natl. Acad. Sci. USA 88:10421 (1991)).

[0077] It should be recognized that a non-naturally occurring plant of the invention, which contains an ectopically expressed nucleic acid molecule encoding an NHX-related gene product, also can contain one or more additional modifications, including naturally and non-naturally occurring mutations that can, for example, increase salt tolerance.

[0078] The invention further provides a method of producing a non-naturally occurring plant characterized by an increased salt tolerance. The method is practiced by ectopically expressing a nucleic acid molecule encoding an NHX-related gene product in the plant, whereby salt tolerance is increased due to ectopic expression of the nucleic acid molecule. In one embodiment, the method is practiced by introducing an exogenous nucleic acid molecule encoding an NHX-related gene product into the plant.

[0079] As discussed above, the term "ectopically" refers to expression of a nucleic acid molecule encoding an NHX-related gene product in a cell type other than a cell type in which the nucleic acid molecule is normally expressed, at a time other than a time at which the nucleic acid molecule is normally expressed or at an expression level other than the level at which the nucleic acid molecule normally is expressed.

[0080] Actual ectopic expression of an NHX-related gene product is dependent on various factors. The ectopic expression can be widespread expression throughout most or all plant tissues or can be expression restricted to a small number of plant tissues, and can be achieved by a variety of routine techniques. Mutagenesis, including seed or pollen mutagenesis, can be used to generate a non-naturally occurring plant, in which a nucleic acid molecule encoding an NHX-related gene product is ectopically expressed. Ethylmethane sulfonate (EMS) mutagenesis, transposon mediated mutagenesis or T-DNA mediated mutagenesis also can be useful in ectopically expressing an NHX-related gene product to produce a seed plant that produces seeds

of increased size (see, generally, Glick and Thompson, supra, 1993). While not wishing to be bound by any particular mechanism, ectopic expression in a mutagenized plant can result from inactivation of one or more negative regulators of NHX, for example.

[0081] Ectopic expression of an NHX-related gene product also can be achieved by expression of a nucleic acid molecule encoding an NHX-related gene product from a heterologous regulatory element or from a modified variant of its own promoter. Heterologous regulatory elements include constitutive regulatory elements, which result in expression of the NHX-related gene product in a limited number of plant tissues.

[0082] Ectopic expression of a nucleic acid molecule encoding an NHX-related gene product can be achieved using an endogenous or exogenous nucleic acid molecule encoding an NHX-related gene product. A recombinant exogenous nucleic acid molecule can contain a heterologous regulatory element that is operatively linked to a nucleic acid sequence encoding an NHX-related gene product. Methods for producing the desired recombinant nucleic acid molecule under control of a heterologous regulatory element and for producing a non-naturally occurring plant of the invention are well known in the art (see, generally, Sambrook et al., supra, 1989; Glick and Thompson, supra, 1993).

[0083] An exogenous nucleic acid molecule can be introduced into a plant for ectopic expression using a variety of transformation methodologies including Agrobacterium-mediated transformation and direct gene transfer methods such as electroporation and microprojectile-mediated transformation (see, generally, Wang et al. (eds), Transformation of Plants and Soil Microorganisms, Cambridge, UK: University Press (1995), which is incorporated herein by reference). Transformation methods based upon the soil bacterium Agrobacterium tumefaciens are particularly useful for introducing an exogenous nucleic acid molecule into a plant. The wild type form of Agrobacterium contains a Ti (tumor-inducing) plasmid that directs production of tumorigenic crown gall growth on host plants. Transfer of the tumor-inducing T-DNA region of the Ti plasmid to a plant genome requires the Ti plasmid-encoded virulence genes as well as T-DNA borders, which are a set of direct DNA repeats that delineate the region to be transferred. An Agrobacterium-based vector is a modified form of a Ti plasmid, in which the tumor inducing

functions are replaced by the nucleic acid sequence of interest to be introduced into the plant host.

[0084] Agrobacterium-mediated transformation generally employs cointegrate vectors or, preferably, binary vector systems, in which the components of the Ti plasmid are divided between a helper vector, which resides permanently in the Agrobacterium host and carries the virulence genes, and a shuttle vector, which contains the gene of interest bounded by T-DNA sequences. A variety of binary vectors are well known in the art and are commercially available, for example, from Clontech (Palo Alto, Calif.). Methods of coculturing Agrobacterium with cultured plant cells or wounded tissue such as leaf tissue, root explants, hypocotyledons, stem pieces or tubers, for example, also are well known in the art (Glick and Thompson, supra, 1993). Wounded cells within the plant tissue that have been infected by Agrobacterium can develop organs de novo when cultured under the appropriate conditions; the resulting transgenic shoots eventually give rise to transgenic plants that ectopically express a nucleic acid molecule encoding an NHX-related gene product. Agrobacterium also can be used for transformation of plants as described in Bechtold et al., C.R. Acad. Sci. Paris. Life Sci. 316:1194-1199 (1993), which is incorporated herein by reference). Agrobacterium-mediated transformation is useful for producing a variety of transgenic plants (Wang et al., supra, 1995) including transgenic plants of the Brassicaceae family, such as rapeseed and flax.

[0085] Microprojectile-mediated transformation also can be used to produce a transgenic plant that ectopically expresses an NHX-related gene product. This method, first described by Klein et al. (Nature 327:70-73 (1987), which is incorporated herein by reference), relies on microprojectiles such as gold or tungsten that are coated with the desired nucleic acid molecule by precipitation with calcium chloride, spermidine or PEG. The microprojectile particles are accelerated at high speed into an angiosperm tissue using a device such as the BIOLISTIC PD-1000 (Biorad; Hercules Calif.).

[0086] Microprojectile-mediated delivery or "particle bombardment" is especially useful to transform plants that are difficult to transform or regenerate using other methods. Microprojectile-mediated transformation has been used, for example, to generate a variety of transgenic plant species, including cotton, tobacco, corn, hybrid poplar and papaya (see Glick

and Thompson, supra, 1993) as well as cereal crops such as wheat, oat, barley, sorghum and rice (Duan et al., Nature Biotech. 14:494-498 (1996); Shimamoto, Curr. Opin. Biotech. 5:158-162 (1994), each of which is incorporated herein by reference). In view of the above, the skilled artisan will recognize that Agrobacterium-mediated or microprojectile-mediated transformation, as disclosed herein, or other methods known in the art can be used to produce a transgenic plant of the invention.

[0087] If desired, a kit of the invention also can contain a plant expression vector. As used herein, the term "plant expression vector" means a self-replicating nucleic acid molecule that provides a means to transfer an exogenous nucleic acid molecule into a plant host cell and to express the molecule therein. Plant expression vectors encompass vectors suitable for Agrobacterium-mediated transformation, including binary and cointegrating vectors, as well as vectors for physical transformation.

[0088] Plant expression vectors can be used for transient expression of the exogenous nucleic acid molecule, or can integrate and stably express the exogenous sequence. One skilled in the art understands that a plant expression vector can contain all the functions needed for transfer and expression of an exogenous nucleic acid molecule; alternatively, one or more functions can be supplied in trans as in a binary vector system for Agrobacterium-mediated transformation.

[0089] In addition to containing a nucleic acid molecule encoding an NHX-related gene product operatively linked to a seed-selective regulatory element, a plant expression vector of the invention can contain, if desired, additional elements. A binary vector for Agrobacterium-mediated transformation contains one or both T-DNA border repeats and can also contain, for example, one or more of the following: a broad host range replicon, an ori T for efficient transfer from *E. coli* to Agrobacterium, a bacterial selectable marker such as ampicillin and a polylinker containing multiple cloning sites.

[0090] A plant expression vector for physical transformation can have, if desired, a plant selectable marker and can be based on a vector such as pBR322, pUC, pGEM and M13, which are commercially available, for example, from Pharmacia (Piscataway, N.J.) or Promega (Madison, Wis.). In plant expression vectors for physical transformation of a plant, the T-DNA borders or the ori T region can optionally be included but provide no advantage.

[0091] The invention will be better understood by reference to the following non-limiting examples.

EXAMPLE 1

Materials and Methods

Plant Material.

Seeds of Brassica napus cv. Westar were rinsed with running water for two days, surface-sterilized with a solution of 10% commercial bleach (0.525% sodium hypochlorite) and 0.1% SDS for 5 min and washed three times with sterile distilled water. Seeds were germinated on Murashige and Skoog medium (MS). Cotyledon explants were excised from 7 day-old seedlings. The binary Ti vector pBI121 was used for transformation. (Jefferson, et al. (1987)) The GUS gene of the binary vector was replaced with the AtNHX1 gene to gain the new expression construct pHZX1. The new construct was electroporated into Agrobacterium tumefaciens strain LBA4404. For co-cultivation, 1 ml of pHZX1 containing LBA4404 Agrobacterium was inoculated into 15 ml LB medium containing 50 mg.l-1 kanamycin, 50 mg.l-1 rifampicin and 200 μM acetone-syringone. The culture was incubated one day at room temperature under constant shaking (250 rpm) and then diluted one time with liquid MS medium. The cotyledon explants were submerged in the Agrobacterium solution for 3 min, blotted on sterile paper towels and returned to the feeder plates for 2 days of co-cultivation. After co-cultivation, the explants were transferred to a selective regeneration medium. (Moloney, et al. (1989)) Regenerated shoots were transferred to fresh medium bi-weekly. When the green shoots were 1-2 cm tall, they were separated from the calli and transferred onto rooting medium which contained modified MS medium supplemented with 3.7 mM KNO3, 4.1 mM NH4NO3, 0.5 mM MgSO4, 75 mg/l Kanamycin, 200 mg/l Ampicillin and 1 mg/l indole butyric acid. Under these conditions, about 98% shoots formed roots in two weeks. Rooted shoots were transplanted to soil, plants were grown and seeds (T1) collected. T1 seeds were grown on MS medium plates containing 15mg/l kanamycin, plants were grown and homozygous seeds (T2) selected. For salt tolerance experiments, wild type and transgenic seeds (T2) overexpressing the vacuolar Na+/H+ antiport were germinated in 250 ml pots containing pro-mix BX peat moss, perlite and vermiculite medium (Premier Brands, New Rochelle, N.Y.) and grown in the

greenhouse. Two weeks after germination the plants were watered bi-weekly with a nutrient solution with low (10 mM) or high (200 mM) concentrations of NaCl. Sixty of each wild-type and transgenic plants were distributed in two groups of thirty plants each, and each group was watered with a solution with low or high salinity. The nutrient solution was obtained by mixing 1.2 g per liter of stock fertilizer (6-11-31, Plant-Prod, Brampton, Ontario) and 1g per liter of Ca(NO3)2. The final nutrient solution contained (in mM) 15 N, 2 P, 6.5 K, 4 Ca, 2 Mg, 9.5 S, micronutrients and was supplemented with 5 mM or 200 mM NaCl. Day temperature was maintained at 28 ± 2 °C and night temperature was 20 ± 2 °C. Relative humidity was maintained at 50 ±10%. Plants were grown under a 14 h/10h light/dark photoperiod. Supplemental lighting consisted of eight high-pressure sodium lamps, and resulted in a total flux (sunlight and supplemental light) of approximately 1,450 μmol $m^{-2}s^{-1}$.

Membrane isolation and Western blots.

[0092] Tonoplast-enriched membrane fractions were isolated from leaves of 10-week-old plants as described. (Zhang, et al. (2001)) Western blots were performed as described.

Leaf, root and seed chemical and lipid analysis.

[0093] Roots were rinsed with distilled water and leaves and roots were collected from fifteen plants from each treatment, pooled in three groups, dried at 70°C for 24 h and the material was ground to a fine powder. Seeds were collected from the rest of the plants 3 weeks later. For the determination of soluble sugars and proline contents, 100 mg of each pool was resuspended in 2 ml of water, sonicated and centrifuged for 10 min at 2,500 xg. Soluble sugar, proline and protein contents were determined in the supernatant as described. (Blumwald, et al. (1985)); Dubois, et al. (1956) and Bates, et al. (1973)) Ion contents were determined by atomic absorption spectrophotometry. Lipids were extracted from 2 g of mature leaf tissue or 3 g of root tissue with chloroform/methanol (2;1,v/v) and purified as previously described. (Williams, et al, (1970)) Lipid classes were separated by thin-layer chromatography (TLC) on silica gel G plates containing ammonium sulfate using acetone/benzene/water (91:30:8,v/v). (Khan, et al. (1977)) The lipids were scraped from the plate and trans-esterified with 1 mL 1.5 M HCl in dry methanol in a microwave oven as previously described and the fatty acid methyl esters (FAME) were extracted from the methanolic HCl with hexane. (Khan, et al, (1993)) Seed oil fatty acid

compositions were determined by direct trans-esterification of whole seeds using the microwave technique. The FAME were analyzed by gas-liquid chromatography using a Hewlett-Packard model 5890 gas-liquid chromatograph (Hewlett-Packard, Mississauga, Ontario, Canada) with a 30 m x 0.25 mm ID DB-23 capillary column (J & W Scientific, Folsom, California) programmed from 160°C to 210°C at 3°C min^{-1}. The FAME were estimated quantitatively using methylpentadecanoate as an internal standard.

Results

[0094] A construct containing the *AtNHX1* was introduced into the genome of *Brassica napus* cv Westar. Sixty-four transgenic plants were obtained and nine homozygous lines from these transgenic plants were obtained in the T2 generation (data not shown). In order to assess whether the enhanced expression of the vacuolar Na^+/H^+ antiport would allow plants to grow in high salt conditions, wild-type and three lines of transgenic plants (with relatively low, medium and high levels of transgene expression) were grown in the presence of 200 mM NaCl (Fig. 5), a concentration that inhibits the growth of almost all crop plants. The overexpression of the vacuolar Na^+/H^+ antiport did not affect the growth of the transgenic plants since similar growth was observed when the wild-type and the transgenic plants were grown in the presence of 10 mM NaCl (Table I). The growth of the wild-type plants was severely affected by the presence of 200 mM NaCl in the growth solution, plant growth was inhibited and the plants were severely stunted (Fig. 5). On the other hand, the transgenic plants grew, flowered and produced seeds (Fig 5, Table I). The growth of the transgenic plants in 200 mM NaCl was correlated with the increased levels of AtNHX1 protein (Fig. 5). Immunoblots of membrane fractions isolated from wild-type and transgenic plants only detected AtNHX1 in the tonoplast-enriched fractions from transgenic plants indicating the proper targeting of the Na^+/H^+ antiport to the tonoplast (Fig. 5).

[0095] We determined the Na^+, K^+, soluble sugars, proline, total protein, nitrogen and phosphorus contents of wild-type and transgenic plants grown at low (10 mM) NaCl and transgenic plants grown at high (200 mM) NaCl (Figs. 6 and 7). At low salinity, no significant differences were seen in the leaf and root Na^+ content from wild-type and transgenic plants (Fig. 6). Dramatic changes were seen in transgenic plants grown at high salinity. A 70- and 9-fold increase in Na^+ content was seen in the leaves and roots of these plants, respectively. The K^+ content of leaves and roots of transgenic plants growing at high salinity decreased by 75% and

82%, respectively. While the leaf soluble sugars content declined during growth at high salinity (Fig. 7), a 6-fold increase in proline content was seen in high-salt grown leaves. There were no significant differences in N (Fig. 7) or total P content (data not shown). It should be noted that a comparison with wild-type plants grown at high salinity was not possible since all of the wild-type plants grown in these conditions were dead.

[0096] The major root and leaf lipids from wild-type grown at low salinity and transgenic plants grown at low and high salinity were analyzed (Table II). No significant differences in the major chloroplastic and extraplastidic lipids were found. The fatty acid composition of the two major extraplastidic lipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) did not differ in either the 16/18C ratio or the degree of unsaturation (not shown). Similarly, no differences were observed in the fatty acid compositions of the chloroplastic lipids digalactosyldiacylglycerol (DGDG) and mongalactosyldiacylglycerol (MGDG). Neither DGDG (synthesized predominantly through the eukaryotic pathway) nor MGDG (synthesized predominantly through the prokaryotic pathway) showed any significant difference in the 16/18C ratio or degree of unsaturation (results not shown). Some differences, however, were seen in the minor chloroplastic lipids, sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) (Fig. 8).

[0097] Although the 16/18C ratios were the same, there were differences in the degree of unsaturation of the 18C fatty acids in both SQDG and PG from transgenic plants grown in 200 mM NaCl. The ratio of palmitic acid (16:0)/trans- Δ^3-hexadecenoic acid (trans16:1) in PG from transgenic plants grown in 200 mM NaCl was significantly higher than in plants grown in 10 mM NaCl.

[0098] In roots, the predominant lipids are the extraplastidic phospholipids. Although the levels of MGDG, synthesized predominantly through the eukaryotic pathway in roots, are similar to those in leaves, the other plastidic lipids are found in very low quantities in roots. There were no significant differences in the fatty acid compositions of PC, PE and MGDG from wild type and transgenic plants grown at 10 mM NaCl or 200 mM NaCl (results not shown). Total fatty acid analyses of the seed oil did not differ significantly in seeds from wild-type plants grown in 10 mM NaCl and transgenic plants grown in 200 mM NaCl (Fig. 9). Quantitatively and

qualitatively the seed oil from the transgenic plants is identical with seed oil from the wild-type plants.

Discussion

[0099] Taken together, our results demonstrate the ability of the transgenic plants to utilize salty water for growth. In spite of the high Na^+ content in the leaves of the transgenic plants grown at 200 mM NaCl, these plants were able to grow, flower and set seed. These results clearly demonstrate that the enhanced accumulation of Na^+, mediated by the vacuolar Na^+/H^+ antiport, allowed the transgenic plants to mitigate the toxic effects of Na^+. (Apse, et al. (1999) and Zhang, et al (2001)) Notably, transgenic plants grown at 200 mM NaCl produced numbers of seeds similar to those of wild-type plants grown at low salinity. Moreover, qualitative and quantitative analyses of the oil content showed no significant differences between seeds from wild-type grown at low salinity and transgenic plants grown at high salinity. It should be noted that although our experiments were carried out in the greenhouse, our results were obtained under growth conditions with a relatively low humidity and high light intensity. The leaf and root K^+ contents of the transgenic plants grown in 200 mM NaCl were lower than those from plants grown in low salinity. Adaptation of plants to saline environments not only depends on their ability to ameliorate the toxic effects of Na^+ *per se,* but also on their ability to overcome salt-induced impaired nutrient acquisition. (Marschner (1995)) This is of particular importance with regards to K^+ uptake and K^+ homeostasis. Potassium concentrations in plant cells are kept under homeostatic control with cytosolic K^+ concentrations in the order of 100 – 200 mM. (Wyn Jones, et al. (1983)) When exposed to relatively low NaCl concentrations, Na^+ ions can promote growth of many plants, in particular at low K^+ concentrations in the growth medium. (Elzam, et al. (1969)) Under high salinity conditions, Na^+ ions may displace K^+ from its carrier binding sites and this competition results in impaired K^+ uptake and lower K^+ cytosolic concentrations. Nevertheless, the growth of the transgenic plants was not significantly affected by high salinity, suggesting that K^+ nutrition was not compromised in our experiments. It should be noted that we have used a high level of K^+ (6.5 mM) in our solutions. Transgenic plants grown in 200 mM NaCl displayed a six-fold increase in proline content compared to plants grown in low salinity. This accumulation of proline in response to high salinity is well documented. Proline contributes to osmotic adjustment, the protection of macromolecules during dehydration, and as a hydroxyl

radical scavenger. (LeRudulier, et al. (1984); Yancey, et al. (1982) and Smirnoff, et al. (1989)) Evidence supporting the role of proline during salt stress was obtained on the basis of salt tolerance in transgenic tobacco plants with enhanced levels of proline biosynthesis and salt tolerance of *Arabidopsis* with suppressed levels of proline degradation. (Kishor, et al. (1995) and Nanjo, et al. (1999)) Moreover, a similar increase in proline content was observed in transgenic tomato plants overexpressing *AtNHX1* growing at high salinity. (Zhang, et al. (2001))

[0100] In all plant cells there are two major sites of lipid synthesis and desaturation of fatty acids. Glycerolipids derived from diacylglycerols synthesized in the extraplastidic compartments of the cell are synthesized by the eukaryotic pathway, whereas lipids derived from diacylglycerol synthesized in plastids are produced by a prokaryotic pathway. (Browse, et al. (1991) and Williams, et al. (2000)) Each compartment possesses different isoforms of glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAT) that show differing specificity toward the fatty acid esterified to the two *sn* positions of the diacylglycerol. In addition, the desaturases of these diacylglycerol are specific to the specific compartment. Thus, through analyses of fatty acid composition it is possible to determine any specific effect of stress on lipid synthesis in the cell compartments. Our data suggest that the major structural lipids of the extraplastidic compartments (PC and PE) and of the chloroplasts (DGDG and MGDG) were unaffected by the overexpression of *AtNHX1* and by the growth of the transgenic plants at high salinity. Only minor changes in the chloroplast lipids, SQDG and PG, were seen in transgenic plants grown in 200 mM NaCl. Little differences in the quantity of lipid or fatty acids were detected in the structural lipids of the cell. The 16/18C ratio remained similar, suggesting little effect on GPAT or LPAT activities. Further, the levels of unsaturation remained constant, indicating little or no effect on the desaturase activity. Only in the minor chloroplast lipids were changes in desaturation seen, the major difference being the 16:0/trans16:1 ratio in PG (1.7 and 1.0 in transgenic plants grown in 200 mM NaCl and plants grown in low salinity, respectively). Previous work has shown that this difference reflects a change in the light-harvesting complexes of the thylakoid membranes during the acclimation of plants to stress. (Huner, et al. (1987)) Our results would suggest that the transgenic plants displayed little signs of stress or acclimation to high NaCl conditions. Analyses of the seed oil show no significant difference between seeds from wild-type and transgenic plants grown at low or high salinity.

[0101] Worldwide, more than 60 million hectares of irrigated land (representing 25% of the total irrigated acreage in the world) have been damaged by salt. (Ghassemi, et al. (1995)) Twenty years ago, Epstein argued for the development of salt tolerant crops with truly halophytic responses to salinity, i.e., accumulation of salt, in which the consumable part is botanically a fruit, such as grain or berries or pomes. (Epstein (1983)) In these plants, Na^+ ions would accumulate mainly in their leaves, and since the water transport to the fruits and seeds is mainly symplastic much of the salt from these organs would be screened. (Ehret, et al. (1986); Lee (1986) and Davies, et al. (2000)) Our results clearly support Epstein's argument. (Epstein (1983)) These results together with the data presented here clearly demonstrate the feasibility of generating salt tolerant crops for agricultural use. Much of the effort towards breeding crop cultivars with improved salt tolerance assumed that salt tolerance will be achieved only after pyramiding several characteristics in a single genotype. (Yeo, et al. and Cuartero, et al. (1999)) However, the modification of a single trait (vacuolar Na^+ accumulation) significantly improved the salinity tolerance of *Brassica* plants. These results strongly suggest that with a combination of breeding and transgenic plants it could be possible to produce salt tolerant crops with far fewer introduced traits than had been anticipated.

EXAMPLE 2

Experimental Protocol

Plant Material and transgenic plants.

[0102] Lycopersicon esculentum (cv Moneymaker) seeds were germinated on Murashige and Skoog medium (MS). Cotyledon explants were excised from 7 day-old seedlings, cut in half and cultured overnight on a one day-old feeder layer consisting of 3 ml of a 7 day-old sugar beet suspension culture plated and overlaid with a sterile Whatman filter paper. The binary Ti vector pBI121 was used for transformation. The GUS gene of the binary vector was replaced with the AtNHX1 gene to gain the new expression construct pHZX1. pHZX1 was electroporated into *Agrobacterium tumefaciens* strain LBA4404. For co-cultivation, 1 ml of pHZX1 containing *Agrobacterium* were inoculated into 15 ml LB medium containing 50 mg/l kanamycin, 50 mg/l rifampicin and 200 μM acetone-syringone. After two days of co-cultivation with *Agrobacterium*, the explants were transferred to selective regeneration medium. (Thomas, et al.

(1981)) Regenerated shoots were transferred to fresh medium bi-weekly. When the green shoots were 1-2 cm tall, they were separated from the calli and transferred onto rooting medium containing modified MS salts. About 98% shoots can form roots in two weeks. Rooted shoots were transplanted to soil and plants regenerated. T1 seeds were grown on plates containing MS medium and 100 mg/l kanamycin and homozygous seeds selected.

[00100] For salt tolerance experiments, wild type and two independent lines (T2) of transgenic plants were grown hydroponically. Seeds were germinated in agar plates containing MS medium under continuous light at 25 °C. Two weeks after germination, sixty of each wild-type and transgenic seedlings were transferred to six hydroponic tanks, containing 20 seedlings each tank, and grown in the greenhouse. Day temperature was maintained at 26 ± 2 °C and night temperature was 22 ± 2 °C. Relative humidity was maintained at 50 ±10%. Plants were grown under a 14 h/10 h light/dark photoperiod. Supplemental lighting consisted of eight high-pressure sodium lamps, and resulted in a total (sunlight and supplemental light) of approximately 1,250 μmol/m2s. The nutrient solution was obtained by mixing 1.2 g per liter of stock fertilizer (tomato fertilizer, Plant-Prod, Brampton, Ontario) and 1g per liter of CaNO3. The final nutrient solution contained (in mg/l) 200 N, 54 P, 256 K, 147 Ca, 42 Mg, micronutrients and was supplemented with 5 mM or 200 mM NaCl. The nutrient solution was replaced every 6 days and the roots were kept under constant aeration.

Membrane isolation and Western blots.

[0100] Membrane fractions were isolated from shoots of 4-week-old plants or tomato fruits from mature plants as described. (Blumwald, et al (1985)) Western blots of the different membrane fractions were performed as described. (Apse, et al. (1999))

Transport assays.

[0101] The cation/H^+ exchange activity was measured by following the pH dependent fluorescence quenching of acridine orange. An acidic-inside pH gradient across the tonoplast vesicles was obtained by activation of the vacuolar H^+-PP_iase. Twenty μg of tonoplast vesicles were added to 0.8 ml buffer containing 0.25 M Mannitol, 5 mM Tris/MES (pH 8.0), 2 mM dithiotreitol, 25 mM KCl, 0.8 mM Tris-PPi and 5 μM acridine orange. Proton translocation was initiated by the addition of 1 mM Mg^{2+} and the change in fluorescence was monitored as

described. (Blumwald, et al. (1985)) When a steady-state pH gradient (acidic inside) was formed, PPi-dependent H^+-transport activity was stopped by the addition of AMDP and the changes in rate of fluorescence recovery were determined in the presence and absence of 50 mM NaCl.

Leaf and fruit chemical analysis.

[0102] Chemical analysis from 3-month old plants was performed. Fully-expanded mature leaves from the six most lower basal nodes (old leaves), developing leaves from the six most upper apical nodes (young leaves), roots and fruits were collected and dried at 70°C for 24 h and the material ground to a find powder. Tomatoes were collected at the mature green/red ripe stage and were allowed one week of further maturation at the bench at room temperature (22 °C) before analysis. For the determination of soluble sugars, 100 mg of each sample was resuspended in 2 ml of water, sonicated and centrifuged for 10 min at 2,500 xg. Soluble sugar and proline contents were determined in the supernatant as described. (Dubois, et al. (1956) and Bates, et al. (1973)) Ion contents were determined by atomic absorption spectrophotometry and chloride content by titration. Water content was calculated as (FW-DW)/FW, where FW and DW are the fresh and dry weight, respectively. Dry weight was obtained by placing the material at 70 °C until a constant weight was obtained. For the determination of soluble solid contents, the tomatoes were strained through a 20 μm mesh and Brix readings of the juice were obtained by refractrometry. Brix readings (°Brix) represent the concentrations of soluble solids as a percentage of total fresh weight.

Results and Discussion

[0103] A construct containing the *Arabidopsis thaliana AtNHX1*, coding for a vacuolar Na^+/H^+ antiport, was introduced into the genome of *Lycopersicon esculentum* cv Moneymaker. Forty-seven transgenic plants were obtained and six homozygous lines from these transgenic plants were obtained in the T2 generation (data not shown). Two of these homozygous lines were used in our experiments. These two lines were chosen because they grew more vigorously in high salinity. The overexpression of the vacuolar Na^+/H^+ antiport did not affect the growth of the transgenic plants (only one line of transgenic plants is shown) since similar growth was observed when the wild-type and the transgenic plants were grown in the presence of 5 mM

NaCl (Figs 1A,B). Immunoblots of membrane fractions isolated from wild-type and transgenic plants only detected AtNHX1 in the tonoplast-enriched fractions from transgenic plants (Fig 1C), indicating the proper targeting of the Na^+/H^+ antiport to the vacuoles. In order to assess whether the enhanced expression of the vacuolar Na^+/H^+ antiport would allow plants to grow in high salt conditions, wild-type and transgenic plants were grown in the presence of 200 mM NaCl, a concentration that inhibits the growth of almost all crop plants. The growth of the wild-type plants was severely affected by the presence of 200 mM NaCl in the growth solution, plant growth was inhibited, most of the plants died or were severely stunted (Fig. 1D). On the other hand, the transgenic plants grew, flowered and produced fruit (Fig 1E).

[0104] To confirm that the presence of the Na^+/H^+ antiport protein resulted in increased Na^+/H^+ exchange, we monitored H^+-dependent Na^+ movements in tonoplast vesicles isolated from leaves. The vesicular lumen was acidified by the activation of the vacuolar H^+-PP_iase in the presence of K^+ ions, since the H^+-PP_iase activity is K^+ dependent. Once the pH gradient was established, the H^+-pump activity was stopped by the addition of AMDP (amino-methylene-diphosphonate), NaCl was added and the rates of Na^+/H^+ exchange measured (Fig. 2A). Tonoplast vesicles isolated from transgenic plants displayed Na^+/H^+ exchange rates 7-fold higher than those from vesicles isolated from wild-type plants. Interestingly, K^+/H^+ exchange was also observed in the tonoplast vesicles after the addition of AMDP, in the absence of external Na^+, (Fig. 2B) and the rates of K^+/H^+ exchange were significantly higher in vesicles isolated from the transgenic plants. These results indicate that the vacuolar Na^+/H^+ antiport was also able to mediate K^+/H^+ exchange, albeit with a lower specificity for K^+ than for Na^+. K^+ ions are involved in a wide number of physiological processes and vacuolar pools generate the turgor needed to drive cell expansion. (Marschner (1995)) Under K^+ deficient growth conditions, vacuolar K^+ concentrations decline while the cytosolic K^+ concentrations remain relatively constant. (Walker, et al. (1996)) Cytosolic K^+ concentrations decline only when the vacuolar K^+ concentrations decrease to values around 20 mM. (Leigh, et al. (1984)) The decrease in cytosolic K^+ concentrations with the concomitant increase in cytosolic Na^+/K^+ ratio is the basis of cytosolic Na^+ toxicity. (Maathuis, et al. (1999)) Given the cytosol-negative electrical potential difference at the tonoplast, an active K^+ translocation mechanism into the vacuole has to be considered. Evidence of a K^+/H^+ antiport was found in tonoplast-enriched fractions from different plants. (Blumwald, et al. (1997)) Although the *Arabidopsis* sequencing project is

completed, only putative K^+/H^+ antiports with similarity to the glutathione-regulated potassium-efflux system of *E. coli* have been sequenced (Accession numbers AAF78418, AAD10158, CCAB80872). (Munro, et al. (1991)) Although their putative function has not yet been characterized in plants, in bacteria and yeast these transporters function as plasma membrane-bound potassium exchangers. (Munro, et al. (1991) and Ramirez, et al. (1998) Although the role of vacuolar Na^+/H^+ antiports in glycophytes has yet to be established, its ubiquity in plants (Blumwald, in preparation) and its ability to mediate K^+ transport would suggest that the vacuolar Na^+/H^+ antiport could also play a role in cellular K^+ homeostasis.

[0105] We determined the ion, sugar, and proline contents of wild-type and transgenic plants grown at low (5 mM) NaCl and two independent transgenic lines grown at high (200 mM) NaCl (Fig. 3). It should be noted that a comparison with wild-type plants grown at high salinity was not possible since all of the wild-type plants grown in these conditions were dead. At low salinity, no significant differences were seen in the content of Na^+ (Fig. 3A), K^+(Fig. 3B), Cl^- (Fig. 3C) soluble sugars (Fig. 3D) or proline (Fig. 3D) of all tissues. Dramatic changes were seen in transgenic plants grown at high salinity. A 28- and 20-fold increase in Na^+ content was seen in fully developed mature (old) and developing (young) leaves, respectively (Fig. 3A), and a similar increase in Cl^- content was also observed (Fig. 3C). The K^+ content of old leaves, young leaves and roots decreased a 5-, 2- and 4-fold, respectively (Fig. 3B). While no significant difference in soluble sugars was observed during growth in high salinity (Fig. 3D), a 3- and 5-fold increase in proline content was seen in leaves and fruits, respectively (Fig. 3E). The accumulation of proline in response to high salinity is well documented. Many prokaryotic and eukaryotic organisms accumulate proline during osmotic and salt stress. (Csonka, et al. (1991) and Schobert (1977)) Proline contributes to osmotic adjustment , the protection of macromolecules during dehydration, and as a hydroxyl radical scavenger. (LeRudulier, et al.; LeRudulier, et al. (1984) and Yancey, et al. (1982)) Evidence supporting the role of proline during salt stress was obtained based on salt tolerance in transgenic tobacco plants with enhanced levels of proline biosynthesis and salt tolerance of *Arabidopsis* with suppressed levels of proline degradation. (Kishor, et al. (1995) and Nanjo, et al. (1999))

[0106] Taken together, our results demonstrate the ability of the transgenic plants to utilize salty water for growth. In spite of the high Na^+ and Cl^- content in the leaves of the transgenic

plants grown at 200 mM NaCl, only a marginal increase in the Na^+ and Cl^- content of the fruits was observed. The K^+ content of the leaves from transgenic plants grown in salt decreased while the K^+ content of the transgenic fruits was higher than the K^+ content of the fruits from plants grown at low salinity. These results clearly demonstrate that the enhanced accumulation of Na^+, mediated by the vacuolar Na^+/H^+ antiport, allowed the transgenic plants to ameliorate the toxic effects of Na^+ and the transgenic plants overcame salt-induced impaired nutrient acquisition. (Rea, et al. (1987)) Notably, transgenic plants grown in the presence of 200 mM NaCl produced fruits (Figs. 4A,B and Table IV). While the transgenic leaves accumulated Na^+ to almost 1% of their dry weight, the fruits displayed only a marginal increase in Na^+ content and a 25% increase in K^+ content. The number of fruits per plant was similar, and although the fruits from the transgenic plants grown in 200 mM NaCl were somewhat smaller, no significant difference was observed in their water content or total soluble solids content (Table IV). The low Na^+ content of the transgenic fruits cannot be due to the lack of vacuolar Na^+/H^+ antiport since the protein was present in the fruit tissue (Fig. 4C). It has been demonstrated that in expanding fruit of many plant species, including tomato, more than 90% of the water transported into the fruit occurs through the phloem. (Ehret, et al. (1986); Lee (1986) and Davies, et al. (2000) Thus the ability to maintain a high cytosolic K^+/Na^+ concentration ratio along the symplastic pathway was most probably responsible for the low Na^+ content of the fruits.

[0107] Worldwide, more than 60 million hectares of irrigated land (representing 25% of the total irrigated acreage in the world) have been damaged by salt. Our findings suggests the feasibility of producing salt tolerant transgenic plants that will produce edible crops.

EXAMPLE 3

Expression of NHX-Related Gene Products in Saccharomyces cerevisiae.

[0108] Expression of NHX-related gene products in yeast is useful to assess and characterize the biochemical properties of the recombinant and native polypeptides. Expression in yeast also facilitates the study of interactions between different NHX-related gene products. Once function has been verified in yeast, the targeting to vacuoles may be verified in plants. We have made conditional expression constructs by ligating the coding region of the AtNHX1 cDNA into two vectors, pYES2 (Invitrogen) and pYEP434. Both constructs provide galactose-inducible

expression, but pYES2 has a URA3 selectable marker while pYEP434 has LEU2 as a selectable marker. Transformation by lithium acetate, 1994), is followed by selection on solid media containing amino acids appropriate for the selection of cells containing the transformation vector. For integrative transformation, the YXplac series of vectors for integrative transformation are used.

[0109] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

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Table I. Plant and seed yield of wild-type (WT) plants grown in the presence of 10 mM NaCl and transgenic plants overexpressing AtNHX1 ($X1OE_1$) grown in the presence of 10 mM and 200 mM NaCl. Each value is the Mean ± SD (n = 15).

	WT	$X1OE_1$	
	(10 mM NaCl)	(10 mM NaCl)	(200 mM NaCl)
Height (cm)	210 ±15	218 ±13	183 ± 17
Fresh Weight (g)	1,750 ±103	1,790 ±110	1,630 ±134
Seeds per plant	470 ±39	481 ± 43	463 ± 35

Table II. Total lipid content of leaves and roots from wild-type (WT) plants grown in the presence of 10 mM NaCl and transgenic plants overexpressing AtNHX1 ($X1OE_1$) grown in the presence of 10 mM and 200 mM NaCl. Each value is the Mean ± SD (n = 5).

		WT	$X1OE_1$	
TISSUE	**LIPID (nmole/gFW)**	**(10 mM NaCl)**	**(10 mM NaCl)**	**(200 mM NaCl)**
LEAVES	**PC**	1,120 ± 538	1,343 ± 375	1,160 ± 287
	PE	670 ±255	814 ± 274	590 ± 214
	SQDG	403 ±103	532 ±109	591 ± 72
	PG	899 ± 70	830 ±181	776 ± 158
	DGDG	1,640 ± 360	1,776 ± 289	1,817 ± 329
	MGDG	4,411 ± 532	4,316 ± 786	3,658 ± 749
ROOTS	**PC**	**844 ± 106**	**688 ± 60**	**826 ± 88**
	PE	**690 ± 110**	**629 ± 60**	**660 ± 56**
	MQDG	**394 ± 92**	**563 ± 83**	**633 ± 50**

Table III.

SEQ ID NO	PROTEIN NUMBER (GI)	PROTEIN ACCESSION	PROTEIN DESCRIPTION (SPECIES)	SEQUENCE
2	NHX1 4324597	AAD16946	NHX1 Na+/H+ exchanger *Arabidopsis thaliana*	1 MLDSLVSKLP SLSTSDHASV VALNLFVALL CACIVLGHLL EENRWMNESI TALLIGLGTG 61 VTILLISKGK SSHLLVFSED LFFIYLLPPI IFNAGFQVKK KQFFRNFVTI MLFGAVGTII 121 SCTIISLGVT QFFKKLDIGT FDLGDYLAIG AIFAATDSVC TLQVLNQDET PLLYSLVFGE 181 GVVNDATSVV VFNAIQSFDL THLNHEAAFH LLGNFLYLFL LSTLLGAATG LISAYVIKKL 241 YFGRHSTDRE VALMMLMAYL SYMLAELFDL SGILTVFFCG IVMSHYTWHN VTESSRITTK 301 HTFATLSFLA ETFIFLYVGM DALDIDKWRS VSDTPGTSIA VSSILMGLVM VGRAAFVFPL 361 SFLSNLAKKN QSEKINFNMQ VVIWWSGLMR GAVSMALAYN KFTRAGHTDV RGNAIMITST 421 ITVCLFSTVV FGMLTKPLIS YLLPHQNATT SMLSDDNTPK SIHIPLLDQD SFIEPSGNHN 481 VPRPDSIRGF LTRPTRTVHY YWRQFDDSFM RPVFGGRGFV PFVPGSPTER NPPDLSKA
3	10716129	BAB16380	Na+/H+ exchanger *Ipomoea nil*	1 MAFGLSSLLQ NSDLFTSDHA SVVSMNLFVA LLCACIVLGH LLEENRWVNE SITALIIGLC 61 TGVVILLLSG GKSSHLLVFS EDLFFIYLLP PIIFNAGFQV KKKQFFVNFM TIMLFGAIGT 121 LISCSIISFG AVKIFKHLDI DFLDFGDYLA IGAIFAATDS VCTLQVLSQD ETPLLYSLVF 181 GEGVVNDATS VVLFNAIQSF DMTSFDPKIG LHFIGNFLYL FLSSTFLGVG IGLLCAYIIK 241 KLYFGRHSTD REVALMMLMS YLSYIMAELF YLSGILTVFF CGIVMSHYTW HNVTESSRVT 301 TRHSFATLSF VAETFIFLYV GMDALDIEKW KFVKNSQGLS VAVSSILVGL ILVGRAAFVF 361 PLSFLSNLAK KNSSDKISFR QQIIIWWAGL MRGAVSIALA YNKFTTSGHT SLHENAIMIT 421 STVTVVLFST VVFGLMTKPL INLLLPPHKQ MPSGHSSMTT SEPSSPKHFT VPLLDNQPDS 481 ESDMITGPEV ARPTALRMLL RTPTHTVHRY WRKFDDSFMR PVFGGRGFVP FVAGSPVEQS 541 PR
4	14039961	AAK53432	Na+/H+ Antiporter *Suaeda maritima* subsp. salsa	1 MLSQLSSFFA SKMDMVSTSD HASVVSMNLF VALLRGCIVI GHLLEENRWM NESITALLIG 61 LSTGIIILLI SGGKSSHLLV FSEDLFFIYL LPPIIFNAGF QVKKKQFFRN FITIILFGAV 121 GTLVSFIIIS LGSIAIFQKM DIGSLELGDL LAIGAIFAAT DSVCTLQVLN QDETPLLYSL 181 VFGEGVVNDA TSVVLFNAIQ NFDLTHIDHR IAFQFGGNFL YLFFASTLLG AVTGLLSAYV 241 IKKLYFGRHS TDREVALMML MAYLSYMLAE LFYLSGILTV FFCGIVMSHY TWHNVTESSR 301 VTTKHAFATL SFVAEIFIFL YVGMDALDIE KWRFVSDSPG TSVAVSSILL GLHMVGRAAF 361 VFPFAFLMNL SKKSNSEKVT FNQQIVIWWA GLMKSAVSVA LAYNQFSRSG HTQLRGNAIM 421 ITSTITVVLF STMVFGLLTK PLILFMLPQP KHFTSASTVS DLGSPKSFSL PLLEDRQDSE 481 ADLGNDDEEA YPRGTIARPT SLRMLLNAPT HTVHHYWRRF DDYFMRPVFG GRGFVPFVPG 541 SPTEQSITNF VTENIS
5	14211574	BAB56105	Na+/H+ Antiporter *Petunia* x *hybrida*	1 MAFDFGTLLG NVDRLSTSDH QSVVSINLFV ALICACIVIG HLLEENRWMN ESITALVIGS 61 CTGIVILLIS GGKNSHILVF SEDLFFIYLL PPIIFNAGFQ VKKKSFFRNF STIMLFGALG 121 TLISFIIISL GAIGIFKKMN IGSLEIGDYL AIGAIFSATD SVCTLQVLNQ DETPLLYSLV 181 FGEGVVNDAT SVVLFNAIQN FDLSHIDTGK AMELVGNFLY LFASSTALGV AAGLLSAYII 241 KKLYFGRHST DREVAIMILM AYLSYMLAEL FYLSAILTVF FSGIVMSHYT WHNVTESSRV 301 TTKHTFATLS FIAEIFIFLY VGMDALDIEK WKFVSDSPGI SVQVSSILLG LVLVGRAAFV 361 FPLSFLSNLT KKTPEAKISF NQQVTIWWAG LMRGAVSMAL AYNQFTRGGH TQLRANAIMI 421 TSTITVVLFS TVVFGLMTKP LIRILLPSHK HLSRMISSEP TTPKSFIVPL LDSTQDSEAD 481 LERHVPRPHS LRMLLSTPSH TVHYYWRKFD NAFMRPVFGG RGFVPFAPGS PTDPVGGNLQ

SEQ ID NO	PROTEIN NUMBER (GI)	PROTEIN ACCESSION	PROTEIN DESCRIPTION (SPECIES)	SEQUENCE
6	14211578	BAB56107	Na+/H+ Antiporter *Torenia hybrida*	1 MGFESVIKLA ASETDNLWSS GHGSVVAITL FVTLLCTCIV IGHLLEENRW MNESIIALII 61 GLATGVIILL ISGGKSSHLL VFSEDLFFIY ALPPIIFNAG FQVKKKSFFR NFATIMMFGA 121 VGTLISFIII SLGTIAFFPK MNMRLGVGDY LAIGAIFAAT DSVCTLQVLS QDETPLLYSL 181 VFGEGVVNDA TSVVLFNAVQ NFDLPHMSTA KAFELVGNFF YLFATSTVLG VLTGLLSAYI 241 IKKLYFGRHS TDREVAIMIL MAYLSYMLAE LFDLSGILTV FFCGIVMSHY TWHNVTENSR 301 VTTKHTFATL SFVAEIFIFL YVGMDALDIE KWRFVSGSMT TSAAVSATLL GLVLLSRAAF 361 VFPLSFLSNL AKKSPLEKIS LRQQIIIWWA GLMRGAVSMA LAYKQFTREG LTVERENAIF 421 ITSTITIVLF STVVFGLMTK PLINLLIPSP KLNRSVSSEP LTPNSITIPL LGESQDSVAE 481 LFSIRGQTSQ GGEPVARPSS LRMLLTKPTH TVHYYWRKFD NAFMRPVFGG RGFVPYVPGS 541 PTERSVRNWE EETKQ
7	14488270	BAB60901	Na+/H+ exchanger *Ipomoea tricolor*	1 MAFGLSSLLQ NSELFTSDHA SVVSMNLFVA LLCACIVLGH LLEENRWVNE SITALIIGLC 61 TGVVILLLSR GKSSHLLVFS EDLFFIYLLP PIIFNAGFQV KKKQFFVNFM TIMLFGAIGT 121 LISCSIISFG AVKIFKHLDI DFLDFGDYLA IGAIFAATDS VCTLQVLSQD ETPLLYSLVF 181 GEGVVNDATS VVLFNAIQSF DMTSFDPKIG LHFIGNFLYL FLSSTFLGVG IGLLCAYIIK 241 KLYFGRHSTD REVALMMLMS YLSYIMAELF YLSGILTVFF CGIVMSHYTW HNVTESSRVT 301 TRHSFATLSF VAETFIFLYV GMDALDIEKW KFVKNSQGLS VAVSSILVGL ILVGRAAFVF 361 PLSFLSNLAK KNSSDKISFR QQIIIWWAGL MRGAVSIALA YNKFTTSGHT SLHENAIMIT 421 STVTVVLFST VVFGLMTKPL INLLLPPHKQ IASGHSSMTT SEPSSPKHFA VPLLDNQHDS 481 ESDMITGPEV ARPTALRMLL RTPTHTVHRY WRKFDDSFMR PVFGGRGFVP FVAGSPAEQS 541 PR
8	4585981	AAD25617	similar to Na+/H+-exchanging proteins *Arabidopsis thaliana*	1 MISPVEHDPQ GQVKQQQAAG VGILLQIMML VLSFVLGHVL RRHRFHYLPE ASGSLLIGLI 61 VGILANISDT ETSIRFCPPP SIPEFSLLSF PRSLVCSFYS VSGRGLISTK SSSSCFCCLP 121 SYYILCFNIC ISSFKFAAAM LCIMDVIFLD IIHLFEPSQV SVFNLNHSFL TLEPLLPLLS 181 SELLSLQLLL VVCYLGGSMY LMYKLPFVEC LMFGALISAT DPVTVLSIFQ VLLLFLLLSV 241 STGYKYSHDV GTDVNLYALV FGESVLNDAV SFYYLLRYWA LPFKTMSLVN RQSSSGEHFF 301 MVVIRFFETF AGSMSAGLAI SFLNSFYTVV FTLLILSEHI VNVMSLFSLF STSIHACRRC 361 WSLRHCFYTL HRNCNRRVMK RYTFSNLSEA SQSFVSSFFH LISSLAETFT FIYMGFDIAM 421 EQHSWSHVGA VNVFGCAYLV NLFRQENQKI PMKHQKALWY SGLRGAMAFA LALQSLHDLP 481 EGHGQIIFTA TTTIVVVTVT FVLLIGGSTG KMLEALEVVG DDLDDSMSEV NSRRSTLISL 541 NIGASSDEDT SSSGSRFKMK LKEFHKTGDG DGDGE

SEQ ID NO	PROTEIN NUMBER (GI)	PROTEIN ACCESSION	PROTEIN DESCRIPTION (SPECIES)	SEQUENCE
9	8515714	AAF76139	putative Na+/H+ antiporter SOS1 *Arabidopsis thaliana*	1 MTTVIDATMA YRFLEEATDS SSSSSSSKLE SSPVDAVLFV GMSLVLGIAS RHLLRGTRVP 61 YTVALLVIGI ALGSLEYGAK HNLGKIGHGI RIWNEIDPEL LLAVFLPALL FESSFSMEVH 121 QIKRCLGQMV LLAVPGVLIS TACLGSLVKV TFPYEWDWKT SLLLGGLLSA TDPVAVVALL 181 KELGASKKLS TIIEGESLMN DGTAIVVFQL FLKMAMGQNS DWSSIIKFLL KVALGAVGIG 241 LAFGIASVIW LKFIFNDTVI EITLTIAVSY FAYYTAQEWA GASGVLTVMT LGMFYAAFAR 301 TAFKGDSQKS LHHFWEMVAY IANTLIFILS GVVIAEGILD SDKIAYQGNS WRFLFLLYVY 361 IQLSRVVVVG VLYPLLCRFG YGLDWKESII LVWSGLRGAV ALALSLSVKQ SSGNSHISKE 421 TGTLFLFFTG GIVFLTLIVN GSTTQFVLRL LRMDILPAPK KRILEYTKYE MLNKALRAFQ 481 DLGDDEELGP ADWPTVESYI SSLKGSEGEL VHHPHNGSKI GSLDPKSLKD IRMRFLNGVQ 541 ATYWEMLDEG RISEVTANIL MQSVDEALDQ VSTTLCDWRG LKPHVNFPNY YNFLHSKVVP 601 RKLVTYFAVE RLESACYISA AFLRAHTIAR QQLYDFLGES NIGSIVINES EKEGEEAKKF 661 LEKVRSSFPQ VLRVVKTKQV TYSVLNHLLG YIENLEKVGL LEEKEIAHLH DAVQTGLKKL 721 LRNPPIVKLP KLSDMITSHP LSVALPPAFC EPLKHSKKEP MKLRGVTLYK EGSKPTGVWL 781 IFDGIVKWKS KILSNNHSLH PTFSHGSTLG LYEVLTGKPY LCDLITDSMV LCFFIDSEKI 841 LSLQSDSTID DFLWQESALV LLKLLRPQIF ESVAMQELRA LVSTESSKLT TYVTGESIEI 901 DCNSIGLLLE GFVKPVGIKE ELISSPAALS PSNGNQSFHN SSEASGIMRV SFSQQATQYI 961 VETRARAIIF NIGAFGADRT LHRRPSSLTP PRSSSSDQLQ RSFRKEHRGL MSWPENIYAK 1021 QQQEINKTTL SLSERAMQLS IFGSMVNVYR RSVSFGGIYN NKLQDNLLYK KLPLNPAQGL 1081 VSAKSESSIV TKKQLETRKH ACQLPLKGES STRQNTMVES SDEEDEDEGI VVRIDSPSKI 1141 VFRNDL
10	9857314	BAB11940	Na/H antiporter Nhx1 *Atriplex gmelini*	1 MWSQLSSLLS GKMDALTTSD HASVVSMNLF VALLCGCIVI GHLLEENRWM NESITALLIG 61 LATGVVILLI SGGKSSHLLV FSEDLFFIYL LPPIIFNAGF QVKKKQFFRN FITIVLFGAV 121 GTLVSFTIIS LGALSIFKKL DIGTLELADY LAIGAIFAAT DSVCTLQVLN QDETPLLYSL 181 VFGEGVVNDA TSVVLFNAIQ SFDLTRIDHR IALQFMGNFL YLFIASTILG AFTGLLSAYI 241 IKKLYFGRHS TDREVALMML MAYLSYMLAE LFYLSGILTV FFCGIVMSHY TWHNVTESSR 301 VTTKHAFATL SFVAEVFLFL YVGMDALDIE KWRFVSDSPG ISVAVSSILL GLVMVGRAAF 361 VFPLSWLMNF AKKSQSEKVT FNQQIVIWWA GLMRGAVSMA LAYNQFTRSG HTQLRGNAIM 421 ITSTISVVLF STMVFGLLTK PLIMFLLPQP KHFTSCSTVS DVGSPKSYSL PLLEGNQDYE 481 VDVGNGNHED TTEPRTIVRP SSLRMLLNAP THTVHHYWRK FDDSFMRPVF GGRGFVPFVP 541 GSPTEQSTNN LVDRT

SEQ ID NO	PROTEIN NUMBER (GI)	PROTEIN ACCESSION	PROTEIN DESCRIPTION (SPECIES)	SEQUENCE
11	NHA1 6323167	NP_013239	Putative Na+/H+ antiporter; Nha1p *Saccharomyces cerevisiae*	1 MAIWEQLEVS KAHVAYACVG VFSSIFSLVS LYVKEKLYIG ESTVAGIFGL IVGPVCLNWF 61 NPLKWGNSDS ITLEITRIVL CLQIFAVAVE LPRKYMLKHW VSVTMLLLPV MTAGWLIIGL 121 FVWILIPGLN FSASLLISAC ITATDPILAQ SVVSGKFAQR VPGHLRNLLS AESGCNDGMA 181 FPFLFLSMNL ILHPGNGREI VKDWICVTIL YECLFGCLLG CFIGYVGRIT IRFAEKKNII 241 DRESFLAFYV VLAFMCAGFG SILGVDDLLV SFAAGATFAW DGWFSQKTQE SNVSTVIDLL 301 LNYAYFIYFG AIIPWSQFNN GEIGTNVWRL IILSIVVIFL RRIPAVMILR PLIPDIKSWR 361 EALFVGHFGP IGVGAIFAAI LARGELESTF SDEPTPLNVV PSKEESKHWQ LIACIWPITC 421 FFIVTSIIVH GSSVAIITLG RHLNTITLTK TFTTHTTNGD NGKSSWMQRL PSLDKAGRSF 481 SLHRMDTQMT LSGDEGEAEE GGGRKGLAGG EDEEGLNNDQ IGSVATSGIP ARPAGGMPRR 541 RKLSRKEKRL NRRQKLRNKG REIFSSRSKN EMYDDDELND LGRERLQKEK EARAATFALS 601 TAVNTQRNEE IGMGGDEEED EYTPEKEYSD NYNNTPSFES SERSSSLRGR TYVPRNRYDG 661 EETESEIESE DEMENESERS MASSEERRIR KMKEEEMKPG TAYLDGNRMI IENKQGEILN 721 QVDIEDRNEA RDDEVSVDST AHSSLTTTMT NLSSSSGGRL KRILTPTSLG KIHSLVDKGK 781 DKNKNSKYHA FKIDNLLIIE NEDGDVIKRY KINPHKSDDD KSKNRPRNDS VVSRALTAVG 841 LKSKANSGVP PPVDEEKAIE GPSRKGPGML KKRTLTPAPP RGVQDSLDLE DEPSSEEDLG 901 DSYNMDDSED YDDNAYESET EFERQRRLNA LGEMTAPADQ DDEELPPLPV EAQTGNDGPG 961 TAEGKKKQKS AAVKSALSKT LGLNK
12	NHX1 6320663	NP_010744	Required for intracellular sequestration of Na+; Nhx1p *Saccharomyces cerevisiae*	1 MLSKVLLNIA FKVLLTTAKR AVDPDDDDEL LPSPDLPGSD DPIAGDPDVD LNPVTEEMFS 61 SWALFIMLLL LISALWSSYY LTQKRIRAVH ETVLSIFYGM VIGLIIRMSP GHYIQDTVTF 121 NSSYFFNVLL PPIILNSGYE LNQVNFFNNM LSILIFAIPG TFISAVVIGI ILYIWTFLGL 181 ESIDISFADA MSVGATLSAT DPVTILSIFN AYKVDPKLYT IIFGESLLND AISIVMFETC 241 QKFHGQPATF SSVFEGAGLF LMTFSVSLLI GVLIGILVAL LLKHTHIRRY PQIESCLILL 301 IAYESYFFSN GCHMSGIVSL LFCGITLKHY AYYNMSRRSQ ITIKYIFQLL ARLSENFIFI 361 YLGLELFTEV ELVYKPLLII VAAISICVAR WCAVFPLSQF VNWIYRVKTI RSMSGITGEN 421 ISVPDEIPYN YQMMTFWAGL RGAVGVALAL GIQGEYKFTL LATVLVVVVL TVIIFGGTTA 481 GMLEVLNIKT GCISEEDTSD DEFDIEAPRA INLLNGSSIQ TDLGPYSDNN SPDISIDQFA 541 VSSNKNLPNN ISTTGGNTFG GLNETENTSP NPARSSMDKR NLRDKLGTIF NSDSQWFQNF 601 DEQVLKPVFL DNVSPSLQDS ATQSPADFSS QNH
13	NHX2 15229877	NP_187154	NHX2 Na+/H+ exchanger *Arabidopsis thaliana*	1 MTMFASLTSK MLSVSTSDHA SVVSLNLFVA LLCACIVIGH LLEENRWMNE SITALLIGLG 61 TGVVILLISR GKNSHLLVFS EDLFFIYLLP PIIFNAGFQV KKKQFFRNFV TIMAFGAIGT 121 VVSCTIISLG AIQFFKKLDI GTFDLGDFLA IGAIFAATDS VCTLQVLNQD ETPLLYSLVF 181 GEGVVNDATS VVLFNAIQSF DLTHLNHEAA FQFLGNFFYL FLLSTGLGVA TGLISAYVIK 241 KLYFGRHSTD REVALMMLMA YLSYMLAELF ALSGILTVFF CGIVMSHYTW HNVTESSRIT 301 TKHAFATLSF LAETFIFLYV GMDALDIEKW RFVSDSPGTS VAVSSILMGL VMLGRAAFVF 361 PLSFLSNLAK KHQSEKISIK QQVVIWWAGL MRGAVSMALA YNKFTRSGHT ELRGNAIMIT 421 STITVCLFST MVFGMLTKPL IRYLMPHQKA TTSTTSMLSD DSTPKSIHIP LLDGEQLDSF 481 ELPGSHQDVP RPNSLRGFLM RPTRTVHYYW RQFDDAFMRP VFGGRGFVPF VPGSPTERSS 541 HDLSKP

SEQ ID NO	PROTEIN NUMBER (GI)	PROTEIN ACCESSION	PROTEIN DESCRIPTION (SPECIES)	SEQUENCE
14	NHX3 15240159	NP_200358	NHX3 Na+/H+ exchanger *Arabidopsis thaliana*	1 MSIGLTEFVT NKLAAEHPQV IPISVFIAIL CLCLVIGHLL EENRWVNESI TAILVGAASG 61 TVILLISKGK SSHILVFDEE LFFIYLLPPI IFNAGFQVKK KKFFHNFLTI MSFGVIGVFI 121 STVIISFGTW WLFPKLGFKG LSARDYLAIG TIFSSTDTVC TLQILHQDET PLLYSLVFGE 181 GVVNDATSVV LFNAVQKIQF ESLTGWTALQ VFGNFLYLFS TSTLLGIGVG LITSFVLKTL 241 YFGRHSTTRE LAIMVLMAYL SYMLAELFSL SGILTVFFCG VLMSHYASYN VTESSRITSR 301 HVFAMLSFIA ETFIFLYVGT DALDFTKWKT SSLSFGGTLG VSGVITALVL LGRAAFVFPL 361 SVLTNFMNRH TERNESITFK HQVIIWWAGL MRGAVSIALA FKQFTYSGVT LDPVNAAMVT 421 NTTIVVLFTT LVFGFLTKPL VNYLLPQDAS HNTGNRGKRT EPGSPKEDAT LPLLSFDESA 481 STNFNRAKDS ISLLMEQPVY TIHRYWRKFD DTYMRPIFGG PRRENQPEC
15	NHX4 15230706	NP_187288	NHX4 Na+/H+ exchanger *Arabidopsis thaliana*	1 MVIGLSTMLE KTEALFASDH ASVVSMNLFV ALLCACIVLG HLLEETRWMN ESITALIIGS 61 CTGIVILLIS GGKSSRILVF SEDLFFIYLL PPIIFNAGFQ VKKKQFFRNF MTIMLFGAIG 121 TLISFVIISF GAKHLFEKMN IGDLTIADYL AIGAIFSATD SVCTLQVLNQ DETPLLYSLV 181 FGEGVVNDAT SVVLFNAIQR FDLTNINSAI ALEFAGNFFY LFILSTALGV AAGLLSAFVI 241 KKLYIGRHST DREVALMMLL AYLSYMLAEL FHLSSILTVF FCGIVMSHYT WHNVTDKSKV 301 TTKHTFAAMS FLAEIFIFLY VGMDALDIEK WDVVRNSPGQ SIGVSSILLG LILLGRAAFV 361 FPLSFLSNLT KSSPDEKIDL KKQVTIWWAG LMRGAVSMAL AYNQFTTSGH TKVLGNAIMI 421 TSTITVVLFS TVVFGLLTKP LVKHLQPSSK QSSTTALQIT LRSSFHDPIL HEPLLSTQGQ 481 SEYDPEQHVS FRMFWKSPSR AIHHYWRKFD NAVMRRIFGG RGVSPVVPGS PIENSVPQWS 541 EEVENKEQNG EP
16	NHX5 30695721	NP_175839	NHX5 Na+/H+ exchanger *Arabidopsis thaliana*	1 MEEVMISPVE HDPQGQVKQQ QAAGVGILLQ IMMLVLSFVL GHVLRRHRFH YLPEASGLIV 61 GILANISDTE TSIRFCPPPS IPEFSLLSFP RSLKPFFSNF GAIVTFAIIG TFVASVVTGG 121 LVYLGGSMYL MYKLPFVECL MFGALISATD PVTVLSIFQD VGTDVNLYAL VFGESVLNDA 181 VSFYYLLRYW ALPFKFFETF AGSMSAEHLF KYAGLDTENL QNLECCLFVL FPYFSYMLAE 241 GVGLSGIVSI LFTGIVMKRY TFSNLSEASQ SFVSSFFHLI SSLAETFTFI YMGFDIAMEQ 301 HSWSHVGFIL FSIVSSFTDR QAVNVFGCAY LVNLFRQENQ KIPMKHQKAL WYSGLRGAMA 361 FALALQSLHD LPEGHGQIIF TATTTIVVVT VLLIGGSTGK MLEALEVVGD DLDDSMSEGF 421 EESDHQYVPP PFSIGASSDE DTSSSGSRFK MKLKEFHKTT TSFTALDKNF LTPFFTTNSG 481 DGDGDGE
17	NHX6 22330742	NP_178079	NHX6 Na+/H+ exchanger *Arabidopsis thaliana*	1 MSSELQISPA IHDPQGQEKQ QQAAGVGILL QIMMLVLSFV LGHVLRRHKF YYLPEASASL 61 LIGLIVGGLA NISNTETSIR FVELFLISFF RHGSISTMSS SFCFCCLPSY YILKIEYLGG 121 VMFLMYRLPF VECLMFGSLI SATDPVTVLS IFQELGSDVN LYALVFGESV LNDADEIVTL 181 LIRSFSFLCC FWQMAISLYR TMSLVRSHSS GQNFFMVIVR FLETFVGSMS AAMKYFILMY 241 SLLLSVYRTW SAVSSYFFHI SRNKTLLFYT SYVSIYFTLI EIVQFVMKHY TYSNLSANSQ 301 RFVSAFFHLI SSLAETFVFI YMGFDIAMEK HSWAANVFGC GYLVNLARPA HRKIPMTHQK 361 ALWYSGKILL CVPLSSYCFY SSVINTKICG FCIGLRGAMA FALALQSVHD LPEGHGQTIF 421 TATTAIVVLT VLLIGGSTGT MLEALEVVGD SHDTSLGDGF EVVNSRYMTS YDDEDTPPGS 481 GFRTKLREFH KSAASFTELD RNYLTPFFTS NNGDYDDEGN MEQHHGNNII L

SEQ ID NO	PROTEIN NUMBER (GI)	PROTEIN ACCESSION	PROTEIN DESCRIPTION (SPECIES)	SEQUENCE
18	NHX7 22325422	NP_178307	NHX7 Na+/H+ exchanger *Arabidopsis thaliana*	1 MTSIIGAALP YKSPEKAIAS SSYSAENDSS PVDAVIFAGT SLVLGTACRY LFNGTRVPYT 61 VVLLVIGIFL GSLEYGTKHN LGKLGHGIRI WNGINPDLLL AVFLPVLLFE SSFSMDVHQI 121 KRCMGQMVLL AGPGVLISTF CLGALIKLTF PYNWDWKTSL LLGGLLGATD PVAVVALLKE 181 LGASKKMTTL IDGESLMNDG VSVVVFQLFF KMVMGHNSDW GSIIKFLVQN SFGAVGIGLA 241 FGIASVFWLK FIFNDTVAQI TVTLSASYFA YYTAQEWAGV SGILTVMILG MFFAAFARTA 301 FKGDSHQSLH HFWYFTTQEM AAYIANTLVF MLSGVIIAES VLSGQTISYK AIKWKFISQF 361 RYGNKAVLQF LFLTGGIVFL TLVVNGSTTQ LLLHLLRMDT LTATKKRILE YTKFEMMKTA 421 LKAFENLGDD EELGSADWPT VIRHISSLKD LEGRQVNPHD GYEAGSLDPT NIMDIRVQAA 481 YWEMLDDGRI TQCTANVLMQ SVDEALDLVS TSSLSDWRGL EPRVHFPNYY KFLQSKIIPH 541 KLVTHLIVER LESACYISSA FLRAHRIARQ QLHIFLGNSN IASTVINESE VEGEEAKQFL 601 EDVRDSFPQV LSVLKTRQVT HYVLNHLNGY IKNLEKVGLL EGKEVSHLHD VVQSDLKKLL 661 RHPPSLKLPN VDDLITSNPL LKDRSSFRSL AIGETDA
19	NHX8 15223849	NP_172918	NHX8 Na+/H+ exchanger *Arabidopsis thaliana*	1 MTSIIGAALP YKSPEKAIAS SSYSAENDSS PVDAVIFAGT SLVLGTACRY LFNGTRVPYT 61 VVLLVIGIFL GSLEYGTKHN LGKLGHGIRI WNGINPDLLL AVFLPVLLFE SSFSMDVHQI 121 KRCMGQMVLL AGPGVLISTF CLGALIKLTF PYNWDWKTSL LLGGLLGATD PVAVVALLKE 181 LGASKKMTTL IDGESLMNDG VSVVVFQLFF KMVMGHNSDW GSIIKFLVQN SFGAVGIGLA 241 FGIASVFWLK FIFNDTVAQI TVTLSASYFA YYTAQEWAGV SGILTVMILG MFFAAFARTA 301 FKGDSHQSLH HFWYFTTQEM AAYIANTLVF MLSGVIIAES VLSGQTISYK AIKWKFISQF 361 RYGNKAVLQF LFLTGGIVFL TLVVNGSTTQ LLLHLLRMDT LTATKKRILE YTKFEMMKTA 421 LKAFENLGDD EELGSADWPT VIRHISSLKD LEGRQVNPHD GYEAGSLDPT NIMDIRVQAA 481 YWEMLDDGRI TQCTANVLMQ SVDEALDLVS TSSLSDWRGL EPRVHFPNYY KFLQSKIIPH 541 KLVTHLIVER LESACYISSA FLRAHRIARQ QLHIFLGNSN IASTVINESE VEGEEAKQFL 601 EDVRDSFPQV LSVLKTRQVT HYVLNHLNGY IKNLEKVGLL EGKEVSHLHD VVQSDLKKLL 661 RHPPSLKLPN VDDLITSNPL LKDRSSFRSL AIGETDA
20	15982204	CAC84522	Na+/H+ antiporter, isoform 1 *Lycopersicon esculentum*	1 MGLDAVARLG VSILSDGDQV SVDSITLFVA LLCGCIVIGH LLEESRWIND SITTLVIGLS 61 TGGIILLTTK GKSSHLLEFD EQLFFIYVLP PIIFNAGFQV KKKQFFRNFV TIMLFGAVGT 121 LISFSIISFG AKELLGKLDI GFLELRDYLA IGAIFSATDS VCTLQALNQD ETPRLYSLVF 181 GEGVVNDATS VVLFNAIQKL DLSHINSRAA LVFTGNFLYL FLASTFLGVL IGLLSAYLIK 241 KIYLGRHSTD REVALMILMA YLSYVMAELF DLSGILTVFI CGIVMSHYTW HNVTFNSKVT 301 TRHAFATLSF IAEIFIFLYV GMDALDIEKW RFVKDSPGKS VGVSAALLGL VLVGRACFVF 361 PLSLFSNCLK RSEHDKFGLK LQVTIWWAGL MRGSVSMALA YNQFTRFGHT QQPGNAVMIT 421 STITIVLFST VVFGLITKPL VRFLLPSSQG FNNLISSEQS FARPLLTNEQ ELELEMGNVD 481 PVRPSGLSIL LKEPSYTIHN HWRRFDDAFM RPLFGGRGFV PDAPELSKGG CDQY

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21	15982206	CAC83608	Na+/H+ antiporter, isoform 2 *Lycopersicon esculentum*	1 MEDHLQISPA GAKAIPGKEQ QAAGYGILLQ IMMLVLSFVI GHVLRRRHFY YIPEASASLL 61 IGLIVGGLAN VSDTETSIRA WFNFHEEFFF LFLLPPIIFQ SGFSLSPKPF FSNFGAIITF 121 AILGTFIASF VTGILVYLGG VTYLMYRLPF VECLMFGALI SATDPVTVLS IFQELGTDVN 181 LYALVFGESV LNDAMAISLY RTMSLVRSHM STDQNYFMIT IRFVETFMGS LSAGVGVGFV 241 SALLFKYAGL DIDNLQNLES CLFVLFPYFS YMLAEGLGLS GIVSILFTGV VMKRYTYPNL 301 SESSQRFVSA FFHLISSLAE TFVFIYMGFD IAMEKHSWSH VGFIFFSILF IVIARAANVF 361 GCAYLVNLVR PPHQKIPAKH QKALWYSGLR GAMAFALALQ PVHDLPEGHG QAIFTATTAI 421 VVLTVLIIGG SAGTMLEALE VVGDGQSGSM DETFEGNNGY IAPSYRDESY DGEPSSGNRF 481 RMKLKEFHKS TTSFSALDKN YLTPFFTTQG GDEDEDEPIM HSSRRAGYDG H
22	5731737	BAA83337	OsNHX1 *Oryza sativa* (japonica cultivar-group)	1 MGMEVAAARL GALYTTSDYA SVVSINLFVA LLCACIVLGH LLEENRWVNE SITALIIGLC 61 TGVVILLMTK GKSSHLFVFS EDLFFIYLLP PIIFNAGFQV KKKQFFRNFM TITLFGAVGT 121 MISFFTISIA AIAIFSRMNI GTLDVGDFLA IGAIFSATDS VCTLQVLNQD ETPFLYSLVF 181 GEGVVNDATS IVLFNALQNF DLVHIDAAVV LKFLGNFFYL FLSSTFLGVF AGLLSAYIIK 241 KLYIGRHSTD REVALMMLMA YLSYMLAELL DLSGILTVFF CGIVMSHYTW HNVTESSRVT 301 TKHAFATLSF IAETFLFLYV GMDALDIEKW EFASDRPGKS IGISSILLGL VLIGRAAFVF 361 PLSFLSNLTK KAPNEKITWR QQVVIWWAGL MRGAVSIALA YNKFTRSGHT QLHGNAIMIT 421 STITVVLFST MVFGMMTKPL IRLLLPASGH PVTSEPSSPK SLHSPLLTSM QGSDLESTTN 481 IVRPSSLRML LTKPTHTVHY YWRKFDDALM RPMFGGRGFV PFSPGSPTEQ SHGGR
23	14211576	BAB56106	Na+/H+ antiporter, *Nierembergia caerulea*	1 MAFDFGTLLG KMNNLTTSDH QSVVSVNLFV ALICACIVIG HLLEENRWMN ESITALVIGS 61 CTGVIILLIS GGKNSHILVF SEDLFFIYLL PPIIFNAGFQ VKKKSFFRNF STIMLFGAVG 121 TLISFIIISA GAIGIFKKMD IGHLEIGDYL AIGAIFAATD SVCTLQVLNQ EETPLLYSLV 181 FGEGVVNDAT SVVLFNAVQN FDLSHISTGK ALQLIGNFLY LFASSTFLGV AVGLLSAFII 241 KKLYFGRHST DREVAIMILM AYLSYMLAEL FYLSGILTVF FCGIVMSHYT WHNVTESSRV 301 TTKHTFATLS FIAEIFIFLY VGMDALDIEK WKFVSDSPGT SIKVSSILLG LVLVGRGAFV 361 FPLSFLSNLT KKNPEDKISF NQQVTIWWAG LMRGAVSMAL AYNQFTRGGH TQLRANAIMI 421 TSTITVVLFS TVVFGLMTKP LILLLLPSQK HLIRMISSEP MTPKSFIVPL LDSTQDSEAD 481 LGRHVPRPHS LRMLLSTPSH TVHYYWRKFD NAFMRPVFGG RGFVPFVPGS PTEPVEPTEP 541 RPAESRPTEP TDE
24	15812035	AAK27314	Na+/H+ exchanger *Citrus* x *paradisi*	1 MDQAISSVVR KLQMVNTSDH NSVVSINIFV ALPCASIVIG HLLEESRWMN ESITALLIGV 61 CAGVIILLTT GGKSSHLFVF SEDLFFIYVL PPIIFNAGFQ VKKKQFFRNF ITIMLFGAIG 121 TLVSCTIISL GVIQFFKKLD IGTLDIGDYL AIGAIFAATD SVCTLQVLNQ DDTPLLYSLV 181 FGEGVVNDAT SVVLFNAIQS FDLTHINTRS AFQFIGNFLY LFFTSTLLGV IGGLLSAYVI 241 KKLYFGRHST DREVAIMVLM AYLSYMLAEL FYLSGILTVF FCGIVMSHYT WHNVTESSRV 301 TTKHTFATLS FVAEIFTFLY VGMDALDIEK WRFVKGSPGT SVAASAMLMG LIMAGRAAFV 361 FPLSFLTNLA KKSPTEKISI KQQVIIWWAG LMRGAVSMAL AYNQFTRSGH TQLRENAIMI 421 TSTITVVLFS TVVFGLMTEP LIRLLLPHPK HTTNHILSDP STPKSLSQPL LEEGQQDSYA 481 DLVGPTVPRP GSLRALLTTP THTVHYYWRK FDDAFMRPVF GGRGFAPFVP GSPTERSVRG 541 GQ

SEQ ID NO	PROTEIN NUMBER (GI)	PROTEIN ACCESSION	PROTEIN DESCRIPTION (SPECIES)	SEQUENCE
25	15027833	AAK76737	Na+/H+ antiporter *Triticum aestivum*	1 MGLDLGALAL KYTGLAVSDH DSIVAINIFI ALLCGCIVFG HLLEGNRWVN ESTTALVLGL 61 ITGGVILICT KGVNSRILIF SEDIFFIYLL PPIIFNAGFQ VKKKQFFRNF ATIILFGAAG 121 TLISFVIITF GAMGLFSKLD VGPLELGDYL AIGAIFSATD SVCTLQVLNQ DEAPLLYSLV 181 FGEGVVNDAT SVVLFNAIQN IDINHFDVFV LLQFIGKFLY LFFTSTVLGV AAGLLSAYII 241 KKLCFARHST DREVAIMILM AYLSYMLSML LDLSGILTVF FCGIVMSHYT WHNVTESSRV 301 TTKHTFATLS FIAEIFLFLY VGMDALDIDK WKLASSSPKK PIALSAVILG LVMVGRAAFV 361 FPLSFLSNLS KKESHPKISF NQQVIIWWAG LMRGAVSIAL AYNKFTTSGH TAVRVNAVMI 421 TSTIIVVLFS TMVFGLLTKP LINLLIPPRP GTAADISSQS FLDPLTASLL GSDFDVGQLT 481 PQTNLQYLLT MPTRSVHRVW RKFDDKFMRP MFGGRGFVPF VPGSPIERSV HGPGLLGTVT 541 EAEDRS
26	28575021	AAK76738	Na+/H+ antiporter *Triticum aestivum*	1 MGYQVVAAQL ARLSGALGTS DHASVVSITL FVALLCACIV LGHLLEENRW LNESITALII 61 GLCTGVVILM TTKGKSSHVL VFSEDLFFIY LLPPIIFNAG FQVKKKQFFR NFMAITLFGA 121 VGTMMSFFTI SLAAIAIFSR MNIGTLDVSD FLAIGAIFSA TDSVCTLQVL NQDETPFLYS 181 LVFGEGVVND ATSVVLFNAL QNFDPNQIDA IVILKFLGNF CYLFVSSTFL GVFTGLLSAY 241 VIKKLYIGRH STDREVALVM LMAYLSYMLA ELLDLSGILT VFFCGIVMSH YTWHNVTESS 301 RVTTKHAFAT LSFIAETFLF LYVGMDALDI EKWKFASDSP GKSIGISSIL LGLVLVGRAA 361 FVFPLSFLSN LTKKTELEKI SWRQQIVIWW AGLMRGAVSI ALAYNKFTRS GHTQLHGNAI 421 MITSTITVVL FSTMLFGILT KPLIRFLLPA SSNGAASDPA SPKSLHSPLL TSQLGSDLEA 481 PLPIVRPSSL RMLITKPTHT IHYYWRKFDD ALMRPMFGGR GFVPYSPGSP TDPNVLVE
27	31580736	AAP55209	Na+/H+ antiporter *Triticum aestivum*	1 MGLDLGALAL KYTGLAVSDH DSIVAINIFI ALLCGCIVFG HLLGGNRWVN ESTAALVLGL 61 ITGGVILICT KGVNSRILIF SEDIFFIYLL PPIIFNAGFQ VKKKQFFRNF ATIILFGAAG 121 TLISFVIITF GAMGLFSKLD VGPLELGDYL AIGAIFSATD SVCTLQVLNQ DEAPLLYSLV 181 FGEGVVNDAT SVVLFNAIQN IDINHFDVFG LLQFIGKFLY LFFTSTVLGV AAGLLSAYII 241 KKLCFARHST DREVAIMILM AYLSCMLSML LDLSGILTVF FCGIVMSHYT WHNVTESSRV 301 TTKHTFATLS FIAEIFLFLY VGMDALDIDK WKLASSSPKK PIALSAVILG LVMVGRAAFV 361 FPLSFLSNLS KKESHPKISF NQQVIIWWAG LMRGAVSIAL AYNKFTTSGH TAVRVNAVMI 421 TSTIIVVLFS TMVFGLLTKP LINLLIPPRP GTAADISSQS FLDPLTASLL GSDFDVGQLT 481 PQTNLQYLLT MPTRSAHRVW RKFDDKFMRP MFGGRGFVPF VPGSPIERSV HGPGLLGTVT 541 EAEDRS
28	30172039	AAP20428	Na+/H+ antiporter NHX1 *Zea mays* subsp. *mays*	1 MGLGVVAELV RLGVLSSTSD HASVVSINLF VALLCACIVL GHLLEENRWV NESTALIVGL 61 GTGTVILMIS RGVVIHVLVF SEDLFFFYLL PPIIFNAGFQ VKKKQFFRNF ITITLFGAVG 121 TLISFTVISL GALGLISRLN IGALELGDYL ALGAIFSATD SVCTLQVLSQ DETPFLYSLV 181 FGEGVVNDAT SVVVFNALQN FDITHIDAEV VFHLLGNFFY LFLLSTVLGV ATGLISALVI 241 KKLYFGRHST DREVALMMLM AYLSYMLAEL FALSGILTVF FGCIVMSHYT WHNVTESSRI 301 TTKHAFATLS FLAETFLFLY VGMDALDIDK WRSVSDTPGK SLAISSILMG LVMVGRAAFV 361 FPLSFLSNLA KKTEHEKISW KQQVVIWWAG LMRGAVSMAL AYKKFTRAGH TQVRGNAIMI 421 TSTIIVVLFS TMVFGLLTKP LINLLIPHRN ATSMLSDDSS PKSLHSPLLT SQLGSDLEEP 481 TNIPRPSSIR GEFLTMTRTV HRYWRKFDDA FMRPMFGGRG FVPFVPGSPT ERNPPDLSKA

SEQ ID NO	PROTEIN NUMBER (GI)	PROTEIN ACCESSION	PROTEIN DESCRIPTION (SPECIES)	SEQUENCE
29	30172041	AAP20429	Na+/H+ antiporter NHX2 *Zea mays* subsp. *mays*	1 MGLGVDAETV RLGVLSSTSD HASVVSNNFF VALLCACIVL GHLLEENRMV NESITALLVG 61 LGTGTVILMI SRGVSIHVLV FSEDLFFIYL LPPIIFNAGF QVKKKQFFRN FITIILFGAI 121 GTLISFVIIS LGAMGLFKKL DVGPLELGDY LAIGAIFSAT DSVCTLQVLN QDETPLLYSL 181 VFGEGVVNDA TSIVVFNALQ NFDITHINAE VVFHLLGNFL YLFLLSTVLG VATGLISALV 241 IKKIYFGRHS TDREVALMML MAYLSYMLAE LFALSGILTV FFGCIVMSHY TWHNVTESSR 301 ITTKHAFATL SFLAETFIFL YVGMDALDIE KWRSVSDTPG KSIAISSILM GLVMLGRAAF 361 VFPLSFLSNL AKKNEHEKIS WKQQVVIWWS GLMRGAVSMA LAYNKFTRAG HTEVRGNEIM 421 ITSTITVVLF STVVFGLLTK PLIRLLMPHR HLTMLSDDST PKSLHSPLLT SQLGSSIEEP 481 TQIPRPTNIR GEFTTMTRTV HRYWRKFDDK FMRPMFGGRG FVPFVPGSPT ERNPHDLSKP
30	32396168	AAP20430	Na+/H+ antiporter NHX3 *Zea mays* subsp. *mays*	1 MSIGLTAETV TNKLASAEHP QVVPNSVFIA LLCLCLVIGH LLEENRWVNE SITAILVGAA 61 TGTVILLISK GKSSHILVFD EELFFIYLLP PIIFNAGFQV KKKQFFRNFI TIILFGAIGT 121 LISFVIISLG AMGLFKKLDV GPLELGDYLA IGAIFSATDS VCTLQVLNQD ETPLLYSLVF 181 GEGVVNDATS VVLFNAVQKI DFEHLTGEVA LQVFGNFLYL FSTSTVLGIA TGLITAFVLK 241 TLYFGRHSTT RELAIMVLMA YLSFMLAELF SLSGIITVFF CGVLMSHVTW HNVTESSRIT 301 SRHVFAMLSF IAETFLFLYV GTDALDFTKW KTSSLSFGKS LGVSSVLLGL VLVGRAAFVF 361 PLSFLSNLSK KHPGEKITIR QQVVIWWAGL MRGAVSIALA FNKFTRAGHT QVRGNAIMIT 421 STIIVVLFST VVFGLLTKPL INLLIPHRNA TSMLSDDSSP KSLHSPLLTS QLISSIEEPT 481 QIPRPTNIRG EFMTMTRTVH RYWRKFDDKF MRPMFGGRGF VPFVPGSPTE RSSPDLSKA
31	32396170	AAP20431	Na+/H+ antiporter NHX4 *Zea mays* subsp. *mays*	1 MGYQVVAAQL KLASSADHAS VVIITLFVAL LCACIVLGHL LEENRWLNES ITALIIGLGT 61 GVVILLISRG KNSRLLVFSE DLFFIYLLPP IIFNAGFQVK KKQFFRNFMT ITLFGAVGTM 121 ISFFTISLGA IATFSRMSIG TLDVGDFLAI GAIFSATDSV CTLQVLHQDE TPFLYSLVFG 181 EGVVNDATSV VLFNAVQKIQ FTHINAWTAL QLIGNFLYLF STSTLLGIGT GLITAFVLKK 241 LYFGRHSTTR ELAIMILMAY LSYMLAELFS LSGLLTVFFC GVLMSHVTWH NVTESSRTTS 301 RHVFATLSFI SETFIFLYVG MDALDFEKWK TSSLSFGGTL GVSGVLMGLV MLGRAAFVFP 361 LSFLSNLAKK HQSEKISFRM QVVIWWAGLM RGAVSMALAL NKFTRSGHTQ LHGNAIMITS 421 TITVVLFSTM VFGMITKPLI RLLLPASGHP RELSEPSSPK SFHSPLLTSQ QGSDLESTTN 481 IVRPSSLRGL LTKPTHTVHY YWRKFDDALM RPVFGGRGFV PFVPGSPTER NPPDLSKA
32	32396174	AAP20432	Na+/H+ antiporter NHX5 *Zea mays* subsp. *mays*	1 MSMGYQVVAA QLKVASSADH ASVVIITLFV ALLCACIVLG HLLEENRWLN ESITALIIGL 61 CTGGVILMTT KGKSSHVLVF SEDLFFIYLL PPIIFIAGFQ VKKKQFFRNF MTITLFGAVG 121 TMISFFTISL GAIAIFSRMN IGTLDVGDFL AIGAIFSATD SVCTLQVLHQ DETPFLYSLV 181 FGEGVVNDAT SVVLFNAVQK IQITHINAEV ALQVFGNFLY LFSTSTLLGI ATGLITSFVL 241 KKLYFARHST TRELAIMMLM AYLSYMLAEL FSLSGILTVF FCGVLMSHVT WHNVTESSRI 301 TSRHVFAMLS FIAETFIFLY VGTDALDFDK WKTSSLSFGG TLGVSALIMA LVLLGRAAFV 361 FPLSVLTNFS NKHENESITF KHQVIIWWAG LMRGAVSIAL AFKQFTYSGV TLDPVNAAMV 421 TNTTIVVLFT TLVFGLLTKP LIRLLMPHRH LTMLSDDSTP KSLHSPLLTS QLGSDLEEPT 481 NIPRPSSIRG EFLTMTRTVH RYWRKFDDAF MRPMFGGRGF VPVVPGSPIE RSVPQWSEEA 541 HNKEP

SEQ ID NO	PROTEIN NUMBER (GI)	PROTEIN ACCESSION	PROTEIN DESCRIPTION (SPECIES)	SEQUENCE
33	32396176	AAP20433	Na+/H+ antiporter NHX6 *Zea mays* subsp. *mays*	1 MGLGVVAELV RLGVLSSTSD HASVVSINLF VALLCACIVL GHLLEENRWV NESITALIIG 61 LCTGVVILLT TKGKSSHILV FSEDLFFIYL LPPIIFNAGF QVKKKQFFRN FMTITLFGAV 121 GTMISFFTIS LGALGLISRL NIGALELGDY LALGAIFSAT DSVCTLQVLS QDETPFLYSL 181 VFGEGVVNDA TSVVVFNALQ NFDITHIDAE VVFHLLGNFF YLFLLSTVLG VATGLISALV 241 IKKLYFGRHS TDREVALMML MAYLSYMLAE LFALSGILTV FFGCIVMSHY TWHNVTESSR 301 ITTKHAFATL SFLAETFLFL YVGMDALDID KWRSVSDTPG KSLAISSILM GLVMVGRAAF 361 VFPLSFLSNL AKKTEHEKIS WKQQVVIWWA GLMRGAVSMA LAYKKFTRAG HTQVRGNAIM 421 ITSTIIVVLF STMVFGLLTK PLINLLIPHR NATSMLSDDS SPKSLHSPLL TSQLGSDLEE 481 PTNIPRPSSI RGEFLTMTRT VHRYWRKFDD AFMRPMFGGR GFVPFVPGSP TERNPPDLSK 541 A
34	22902099	AAM54141	Na+/H+ antiporter *Gossypium hirsutum*	1 MVAPQLAAVF TKLQTLSTSD HASVVSMNIF VALLCACIVI GHLLEENRWM NESITALIIG 61 VFTGVIILLT SGGKSSHLLV FSEDLFFIYL LPPIIFNAGF QVKKKQFFRN FITIMLFGAV 121 GTLISCTIIS LGVINFFKEM DIGSLDIGDF LAIGAIFAAT DSVCTLQVLN QDETPLLYSL 181 VFGEGVVNDA TSVVLFNAIQ SFDLVNTSPR ILLEFIGSFL YLFLASTMLG VIVGLVSAYI 241 IKKLYFGRHS TDREFALMML MAYLSYIMAE LFYLSGILTV FFCGIVMSHY TWHNVTESSR 301 VTTKHAFATL SFVAETFLFL YVGMDALDME KWRFVSDSPG TSVAVSAVLM GLVMVGRAAF 361 VFPLSFLSNL AKKSTSEKIS FREQIIIWWA GLMRGAVSMA LAYNQFTRGG HTQLRGNAIM 421 ITSTITIVLF STVVFGLMTK PLIRFLLPHP KPTASMLSDQ STPKSMEAPF LGSGQDSFDD 481 SLIGVHRPNS IRALLTTPAH TVHYYWRKFD NAFMRPMFGG RGFVPFVPGS PTERSEPNLP 541 QWQ
35	30144703	AAP15178	Na+/H+ antiporter *Suaeda maritima* subsp. *salsa*	1 MWSQLSSFFA SKMDMVSTSD HASVVSMNLF VALLCGCIVI GHLLEENRWM NESITALLIG 61 LSTGIIILLI SGGKSSHLLV FSEDLFFIYL LPPIIFNAGF QVKKKQFFRN FITIILFGAV 121 GTLVSFIIIS LGSIAIFQKM DIGSLELGDL LAIGAIFAAT DSVCTLQVLN QDETPLLYSL 181 VFGEGVVNDA TSVVLFNAIQ NFDLTHIDHR IAYRIAFQFG GNFLYLFFAS TLLGAVTGLL 241 SAYVIKKLYF GRHSTDREVA LMMLMAYLSY MLAELFYLSG ILTVFFCGIV MSHYTWHNVT 301 ESSRVTTKHA FATLSFVAEI FIFLYVGMDA LDIEKWRFVS DSPGTSVAVS SILLGLLMVG 361 RALLFSLVFL MNLSKKSNSE KVTFNQQIVI WWAGLMRGAV SVALAYNQFS RSGHTQLRGN 421 AIMITSTITV VLFSTMVFGL LTKPLILFML PQPKHFTSAS TVSDLGSPKS FSLPLLEDRQ 481 DSEADLGNDD EEAYPRGTIA RPTSLRMLLN APTHTVHHYW RRFDDYFMRP VFGGRGFVPF 541 VPGSPTEQST TNLSQRT
36	28201131	BAC56698	Na+/H+ antiporter *Hordeum vulgare*	1 MAFEVVAAQL ARLSDALATS DHASVVSINL FVALLCACIV LGHLLEENRW LNESITALII 61 GLCTGVVILM TTKGKSSHVL VFSEDLFFIY LLPPIIFNAG FQVKKKQFFR NFMTITLFGA 121 VGTMISFFTI SLAAIAIFSK MNIGTLDVSD FLAIGAIFSA TDSVCTLQVL NQDETPFLYS 181 LVFGEGVVND ATSVVLFNAL QNFDPNQIDA IVILKFLGNF CYLFVSSTFL GVFSGLLSAY 241 IIKKLYIGRH STDREVALMM LMAYLSYMLA ELLDLSGILT VFFCGIVMSH YTWHNVTESS 301 RVTTKHAFAT LSFIAETFLF LYVGMDALDI EKWKFASDSP GKSIGISSIL LGLVLVGRAA 361 FVFPLSFLSN LTKKTELEKI SWRQQIVIWW AGLMRGAVSI ALAYNKFTRS GHTQLHGNAI 421 MITSTITVVL FSTMLFGILT KPLIRFLLPA SSNGDPSEPS SPKSLHSPLL TSMLGSDMEA 481 PLPIVRPSSL RMLITKPTHT IHYYWRKFDD ALMRPMFGGR GFVPYSPGSP TDPNVIVA

SEQ ID NO	PROTEIN NUMBER (GI)	PROTEIN ACCESSION	PROTEIN DESCRIPTION (SPECIES)	SEQUENCE
37	27948863	AAO25547	Na+/H+ antiporter *Hordeum brevisubulatum*	1 MGWGLGDPPA DYGSIMAVGL FVALMCICII VGHLLEENRW MNESTTALLL GLGAGTVILF 61 ASSGKNSRLM VFSEDLFFIY LLPPIIFNAG FQVKKKQFFR NFMTITLFAV VGTLISFSII 121 SLGAMGLISR LNIGALELGD YLALGAIFSA TDSVCTLQVL SQDETPFLYS LVFGEGVVND 181 ATSVVLFNAI QNFDLGNFSS LKFLQFIGNF LYLFGASTFL GVASGLLSAY VIKKLYFGRH 241 STDREVAIMM LMAYLSYMLA ELLDLSGILT VFFCGIVMSH YTWHNVTESS RVTTKHAFAT 301 LSFISETFLF LYVGMDALDI EKWKIVSETY SPMKSITLSS IILALVLVAR AAFVFPLSYL 361 SNLTKKTAGE KISIRQQVII WWAGLMRGAV SIALAYNKFA KSGHTQLPSN AIMITSTIII 421 VLFSTIVFGL LTKPLIRLLI PARHLTREVS ALSEPSSPKS FLEQLTVNGP ETDVENGVSI 481 RRPTSLRMLL ASPTRSVHHY WRKFDNAFMR PVFGGRGFVP FVPGSPTESS VPLLAHGSEN
38	29825705	AAO91943	Vacuolar Na+/H+ antiporter *Hordeum vulgare*	1 MGPDLGALAL RYTGLAVSDH DSIVAINIFI ALLCGCIVFG HLLEGNRWVN ESTTAIVLGL 61 ITGGVILLCT KGVNSRILIF SEDIFFIYLL PPIIFNAGFQ VKKKQFFRNF ATIILFGAVG 121 TLISFVIITL GAMGLFRKLD VGPLELGDYL AIGAIFSATD SVCTLQVLNQ DQAPLLYSLV 181 FGEGVVNDAT SVVLFNAIQN IDLNHFDVLV LLQLIGKFLY LFLTSTVLGV AAGLLSAYII 241 KKLCFARHST DREVAIMILM AYLSYMLSML LDLSGILTVF FCGIVMSHYT RHNVTESSRV 301 TTKHTFATLS FIAEIFLFLY VGMDALDIDK WKLASSSPKK PIALSAVILG LVMVGRAAFV 361 FPLSYLSNLS KKESHPKISF NQQVIIWWAG LMRGAVSIAL AYNKYTTSGH TAVRVNAVMI 421 TSTIIVVLFS TMVFGLLTKP LINLLVPPRP GTAADISSQS FLDPLTASLL GSDFDVGQLT 481 PQTNLQYLLT MPSRSVHRVW RKFDDKFMRP MFGGRGFVPF VPGSPIERSV HGPGLLGTVT 541 EAENRS

Table IV. Plant and fruit yield of wild-type (WT) tomato plants grown in the presence of 5 mM NaCl and T2 transgenic plants overexpressing AtNHX1 (OEX1) grown in the presence of 5 mM and 200 mM NaCl. Plants were harvested 12 weeks after germination. Each value is the Mean ± SD (n = 10 individual plants).

	WT	OEX1	
	(5 mM NaCl)	(5 mM NaCl)	(200 mM NaCl)
Height (cm)	124.0±8.2	128.8±9.5	107.6±5.2
Fresh Weight (g) (without fruit)	1,270±103	1,329±110	1,123±134
Fruit per plant	17.2±1.3	17.8±.6	18.4±1.5
Fruit weight (g)	119.5±13.4	116.7±9.0	105.7±6.7
Fruit water content(%)	90.8±3.2	90.2±2.2	90.7±2.3
Solid solute content (^{o}Brix)	4.2±0.6	4.4±0.7	4.2±0.5

Table V. Relative yield decrease of representative plants.

	RELATIVE YIELD DECREASE			
	25%		50%	
CROP	(mmho/cm)	(mM NaCl)	(mmho/cm)	(mM NaCl)
Barley	13	120	18	170
Sugarbeet	11	105	15	150
Sorghum	7.2	65	11	100
Soybean	6.2	59	7.5	65
Rice	3.8	36	5.9	50
Corn	3.8	36	5.9	50
Alfalfa	5.4	45	8.8	75
Cucumber	4.4	40	7.0	65
Potato	2.8	36	5.9	50
Beans	2.3	18	3.2	28
Grape	4.1	37	6.7	62
Orange	3.2	28	4.8	43
Peach	2.9	25	4.1	35
Strawberry	1.8	14	2.5	21